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STUDIES ON A NEW SERIES OF SYNTHETIC ESTROGENIC SUBSTANCES

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SINCE 1938 when Dodds and his co-workers first reported the estrogenic potency of stilbestrol, papers have accumulated reporting the physiologic action of stilbestrol, hexestrol and their derivatives. This work has been reviewed adequately by von Haam, *et al.* (1941); Morrell and Hart (1941) and the Council on Pharmacy and Chemistry (1942).

Shortly after stilbestrol was released for clinical trial reports of toxicity began to appear. The percentages of side reactions varied with the clinic groups, but were sufficiently high to make many clinicians hesitant in its use. In 1939 a program of investigation was undertaken in this laboratory with the purpose of developing a synthetic estrogen which would be clinically effective in small doses, when given orally, but would be free from the 'toxicity' reported for stilbestrol. The chemical aspects of this problem are being reported elsewhere by Stuart and Tallman.

It was decided not to attempt to modify the stilbestrol molecule, but to endeavor to synthesize a physiologically active compound of different fundamental structure. It will be remembered that it is possible to write the structural formula for stilbestrol in such a way as to resemble the spatial structure of estradiol. While it was felt that this similarity was probably a coincidence, nevertheless, the hypothesis of necessary spatial similarity seemed a logical starting point in the synthesis of a new estrogen. Hence, one of the first compounds made was, 1-(meta-hydroxyphenyl)-3-(para-hydroxyphenyl) hexane, (A, fig. 1) which may be written to resemble estradiol, (B, fig. 1). This compound is estrogenic, but not strongly so, 20 mg. representing one Allen-Doisy rat unit.

Further work with the more potent compounds reported here made it evident that spatial resemblance to the natural estrogen had little obvious relation to activity, and that the ethylene and ethane linkages of stilbestrol and hexestrol were not prerequisite to high estrogenic potency. The work was continued with synthesis of many mono, di, and tri-substituted com-

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pounds derived from 1,3-di (para-hydroxyphenyl) propane, having the basic formula shown in C of figure 1. In these compounds one or two of the substituents, R_1, R_2, or R_3, may be hydrogen. To date 134 such compounds have

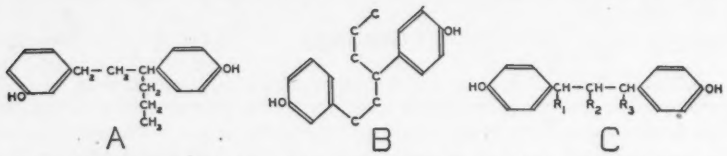

Fig. 1. *A. Compound 29.* 1-(meta-hydroxyphenyl)-3-(para-hydroxyphenyl) hexane. Twenty mg. gives a positive estrous response. *B. Compound 29* showing the similarity of spatial configuration to estradiol. (Carbon skeleton only shown.) *C.* Basic formula for all synthetic estrogens discussed in this report. Substituents, R_1, R_2, R_3, may be hydrogens, or organic radicals.

been made and tested for estrogenic activity, and the potency of the more active members of each group is reported here.

The Allen-Doisy technique has been used for assay and the figures reported in table 1 represent the minimum amount of material which will

TABLE 1. ACTIVITY OF A SERIES OF SYNTHETIC ESTROGENS, HAVING THE BASIC FORMULA SHOWN IN C IN FIGURE 1, AND HAVING SUBSTITUENTS AS SHOWN HERE. POTENCY IS EXPRESSED AS THE MINIMUM AMOUNT WHICH WILL PRODUCE FULLY CORNIFIED SMEARS IN 70 PER CENT OF THE GROUP OF CASTRATED RATS

COMPOUND NUMBER	CHEMICAL NAME	SUBSTITUENTS			MINIMUM DOSE, MICROGRAMS
		R_1	R_2	R_3	
32	1,3-di (p-hydroxyphenyl) hexane	$-H$	$-H$	$-C_3H_7$	2000
82	1,3-di (p-hydroxyphenyl) 2-propyl propane	$-H$	$-C_3H_7$	$-H$	5000
48	3,5-di (p-hydroxyphenyl) heptane	$-C_2H_5$	$-H$	$-C_2H_5$	1000
105	1,3-di (p-hydroxyphenyl) 2-ethyl pentane	$-H$	$-C_2H_5$	$-C_2H_5$	15
102	2,4-di (p-hydroxyphenyl) 3-ethyl pentane	$-CH_3$	$-C_2H_5$	$-CH_3$	10
112	2,4-di (p-hydroxyphenyl) 3-propyl pentane	$-CH_3$	$-C_3H_7$	$-CH_3$	8
118	2,4-di (p-hydroxyphenyl) 3-ethyl hexane	$-CH_3$	$-C_2H_5$	$-C_2H_5$	3
119	2,4-di (p-hydroxyphenyl) 3-ethyl heptane	$-CH_3$	$-C_2H_5$	$-C_3H_7$	15
120	2,4-di (p-hydroxyphenyl) 3-propyl hexane	$-CH_3$	$-C_3H_7$	$-C_2H_5$	12

produce fully cornified vaginal smears in 70 per cent of the rats used for the test. At least 20 rats were used for each assay. The material was given in a single dose in 0.2 cc. of sesame oil and smears were made at intervals beginning 48 hours after injection. Compounds *102*, *105*, *112*, *118*, *119* and *120* were sufficiently active to warrant further investigation. Long term experiments were set up to determine the effect of continued administration of these compounds, in varying dosages, to laboratory animals. Careful study showed that these substances had complete estrogenic activity. Castration changes in the pituitary were repaired, the priming action prior to the administration of progesterone was normal, and large overdoses gave results indistinguishable from those reported by others and found by us to follow similar treatment with natural estrogens.

As may be recognized, many of the compounds listed in the table may exist in several optically isomeric forms, and considerable difference in

physiologic activity between such isomers might be expected. Chemical investigations, which will be reported fully elsewhere, made it possible to test certain of these isomers, and one of these—an isomer of *118*—was found to be extremely active, giving a positive estrous response at a level of 0.8 micrograms. This substance is approximately 2.5 times more active than estrone. With this compound, *118B*,[1] extensive physiologic and clinical work is being done. Freed, Eisen and Greenhill (1942) and Taylor and Thompson (1942) have reported clinical work, and preliminary reports of the physiologic investigations have been presented by Blanchard (1942) and Stebbins and Blanchard (1942).

SUMMARY

A new series of synthetic estrogens, unrelated to stilbestrol is reported, and the estrogenic activities of the more potent compounds are given. One of these is sufficiently active to warrant intensive laboratory and clinical trial.

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[1] The compound *118B* is now on the market under the name of Octofollin.

EFFECT OF ADRENAL CORTICAL COMPOUNDS ON EDEMA FORMATION OF FROGS' HIND LIMBS[1]

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THE METHOD of Cartland and Kuizenga (1936) for the assay of adrenal cortical extract depends on the ability of the test material to maintain the life of adrenalectomized rats; it requires the use of relatively large quantities of extract and a period of several weeks for each determination. A somewhat more rapid and sensitive method is that of Selye and Schenker (1938) which makes use of the changes in the acute response of adrenalectomized rats to temperature fluctuations. This procedure requires moderate quantities of the fluid to be tested and a period of 24 hours for its completion.

In the course of perfusion studies on isolated frog's legs (Loewen-Trendelenburg preparation) it was found that small concentrations of adrenal cortical extracts in the perfusate markedly affect the development of edema in the legs. The rate of the edema formation, measured by the successive changes in weight, was found to serve as a quantitative means for determining the relative concentration of the extracts in the perfusate. The advantage of the method lies in the rapidity with which results are obtained and the precision in determining extremely small concentrations. Furthermore, the exclusion of all tissues except muscle and skin in the preparation used for the perfusion reduces to a minimum the possibility of intermediary secretions being supplied to the perfusion fluid which might affect the assay of the substances tested.

It should be noted that the method presented in this paper deals with a biologic effect, primarily, of changes in the rate of edema formation and is not to be regarded as a measure of general adrenal cortical activity. The observed changes in the rate of edema formation might be considered a function of capillary permeability. This would be in keeping with statements in the literature that adrenal cortical substances possess the property of reducing capillary permeability. However, before deciding on the site of action several features must be taken into account which are considered in the discussion. In any event, the method serves satisfactorily as a means of assay.

METHOD

The rates of edema formation are determined from a continuous kymographic record of the weight of the perfused hind-limbs of a frog. The prepa-

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[1] Aided by a grant from the Josiah Macy Jr. Foundation. A preliminary report was made at the twenty-sixth annual meeting of the Association for the Study of Internal Secretions, June 9, 1942, at Atlantic City, N. J.

ration is made by transecting a medium sized frog immediately posterior to the forelimbs and removing all of the abdominal viscera, except the kidneys. This procedure cuts through the aorta and the posterior vena cava coming from the kidneys but leaves the renal portal system intact. A fine metal cannula (shank of a 25-gauge hypodermic needle, 1.5 cm. in length) connected with the perfusion system is then inserted into the aorta close to its bifurcation into the iliac arteries. Immediately after cannulation the perfusion is begun. The time between transection of the frog and the start of perfusion should not exceed two minutes, which is less than the normal clotting time of amphibian blood, and assures the removal of all the blood at the onset of perfusion.

The perfusion medium passes through the vessels of the legs and returns by way of the renal portal system through the kidneys. The resistance offered by the capillary bed of the kidneys serves to maintain a venous pressure in the muscles and skin of the legs.

The legs are now inserted, feet first, into a light, cylindrical, slightly tilted chamber open at the lower end. It is lined with moist filter paper and suspended by a helical phosphor-bronze spring, the vertical movements of which are recorded by means of a light heart lever on a slowly moving kymograph drum. The connection between the cannula in the aorta and the perfusion system is a 6-inch length of latex capillary tubing, with a lumen into which a 25-gauge needle fits tightly.[2] The thinness of the tubing permits a maximum flexibility and lightness of the connection for the free movement of the suspended moist chamber. The extension of the helical spring with change in weight of the preparation is linear and, with the magnification of the heart-lever, is of such a magnitude that a 1-gm. change in weight of the preparation will cause a displacement of about one cm. on the kymograph drum which moves at the rate of .5 cm./min.

The perfusion fluid, after passing through the preparation, falls from the open end of the chamber in a series of drops approximately constant in size. The fall of each drop causes a momentary decrease in weight which is recorded as a displacement of the weight curve, giving to the line a serrated appearance. Changes in rate of flow through the preparation, by inducing changes in the frequency of drop formation, are indicated on the drum by the spacing of the serrations on the line. The net change in weight of the preparation during a perfusion appears on the record as the slope of the line.

The fluid to be perfused is forced out of the storage reservoir and at the same time is oxygenated by a compressed gas consisting of a mixture of 1.5 per cent carbon dioxide and 98.5 per cent oxygen, the perfusion pressure being kept constant at 20 ± 2 mm. Hg by an adjustable mercury regulator.[3] The fluid on its way to the cannula from the reservoir passes through a drop-

[2] This tubing was purchased from the Miller Rubber Co., Akron, Ohio.

[3] The perfusion pressure used by Richards and his co-workers was 35 mm. Hg. Danielli, who used a preparation similar to ours, states that he maintained the perfusion pressure at 14. We used a pressure of 20 mm. Hg, which was found to meet the requirements of the experiments. We are continuing to investigate the effects of perfusion pressure.

ping chamber (modified Murphy drip) which serves as a trap to eliminate gas bubbles and to which a manometer is connected. The perfusate passes through the capillary rubber-tubing and cannula into the aorta of the preparation. The various parts of this perfusion system are shown diagrammatically in figure 1.

The method of making an assay depends on the measurement of changes in the rate of edema formation caused by the addition to the perfusate of a given substance. An edema formation rate is first obtained by perfusing the preparation with a control perfusate for one hour. This is followed by a

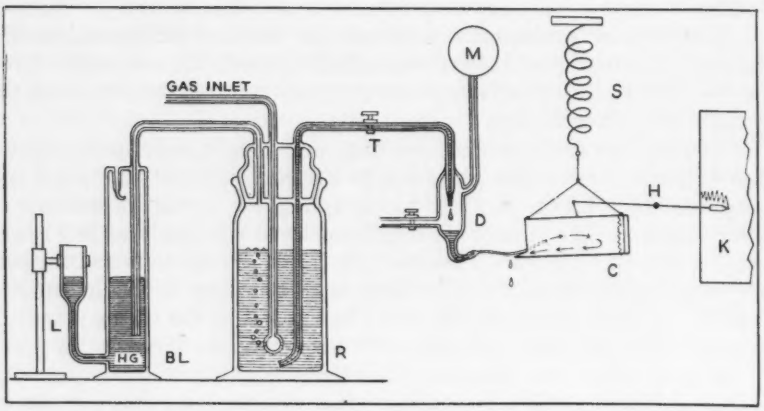

Fig. 1. Diagram of perfusion apparatus. *Bl*, pressure-regulating mercury safety valve with adjustable leveling bulb, *L*; *R*, perfusion reservoir; *T*, 3-way stop-cock connected to duplicate reservoir; *D*, dropping chamber; *M*, manometer; *C*, moist chamber (arrows indicate direction of flow in limb); *S*, phosphor-bronze spring; *H*, heart lever; *K*, kymograph.

perfusion during the next hour with the control perfusate to which has been added a measured quantity of the substance to be tested. The control and experimental perfusates are placed in separate reservoirs, either of which may be connected for perfusion by changing a stop-cock.

The control perfusate was a 0.25 per cent solution of Eastman purified, calf-skin gelatin[4] in amphibian Ringer's solution, buffered to pH 7.6 with acid phosphate and bicarbonate. Its crystalloid osmotic pressure is isotonic with amphibian blood. On the other hand the colloid osmotic pressure is one-fourth that required to prevent edema. This colloidal dilution is made in order to assure a measurable rate of edema formation. This rate is compared with any decrease in the rate of edema formation produced by a solution containing the substance to be tested.

During the first 10 minutes of the perfusion period there is, usually, a rapid and variable increase in weight of the preparation, caused by filling up of the partially collapsed vessels by the perfusing fluid. After this period,

[4] Available from Eastman Kodak Co., Rochester, N. Y., catalog #1099.

however, the rate of increase in weight becomes constant and serves as a measure of the retention in the tissues of fluid escaping from the vascular system. The perfusion is continued for the remainder of the first hour. The experimental perfusate is then connected with the preparation and the perfusion continued for a second hour.

Two major irregularities are sometimes met with which invalidate the preparation. They deal with deviations of the observed rate of edema formation during the control period. The rate may be too small for accurate assay

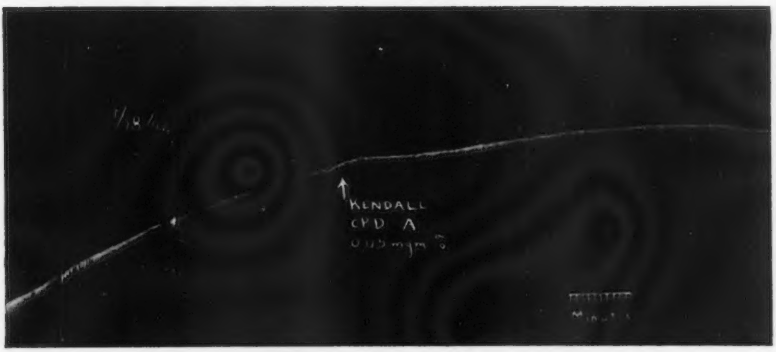

Fig. 2. Photographic representation of a typical kymograph record.

or it may be inconstant and therefore indeterminate.[5] Preparations which show these must be discarded.

Figure 2 is a photographic representation of a satisfactory record. Figure 3 is a diagrammatic representation of a typical kymograph tracing by which the slopes of the two sections of the recorded weight curve are compared. The slope of the initial portion of the curve, as control, is represented by the ratio OS/OI. The slope of the second portion of the line, obtained after the introduction of the compound to be tested, is represented by OC/OI. The ratio of the experimental slope value, divided by that of the control medium becomes OS/OC which is the measure of the efficacy of the substance tested in altering the rate of edema formation.

A fresh frog-leg preparation is used for each assay lasting over a period of 2 hours which is well within the limit for maintaining normal conditions. We have carried control perfusions for longer than 4 hours with no alterations of the rate of edema formation. If the precautions presented in this section are observed, two independent assays of a sample to be tested should agree within 20 per cent.

[5] The rate of edema formation during the control period must be at least 1 gm. in 10 minutes. A lower base rate may be ascribed to *a*) poor condition of the frog. *b*) Insufficient rate of flow from the perfusion apparatus, due either to insufficient pressure or to clots or debris in the tubing leading to the cannula. Any rate of flow less than 1 cc. per minute is to be regarded with suspicion. *c*) Errors in preparation of the perfusate. The concentration of the gelatin should not exceed 0.25 per cent. The cause of nonline rity probably lies within the frog. In our experience not more than 40 per cent of the preparations were discarded because of the above irregularities.

RESULTS

Effect of whole cortical extract. Kendall's whole cortical extract,[6] and two commercial products, were used. It was found that all three greatly reduced the rate of edema formation. The comparison of the several whole extracts was made by reference to a calibration curve. This curve was prepared by determining the ratios of the lowering of the rate of edema formation for several different concentrations of a single extract.

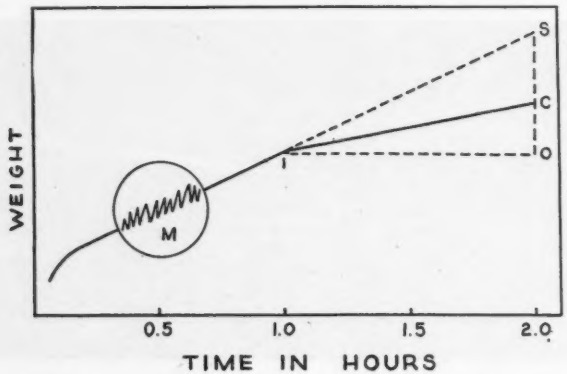

Fig. 3. Diagram of a typical kymograph record. *Ordinates*, represent arbitrary units; *abscissa*, time in hours. At point, *I*, control medium was replaced by experimental. Insert, *M*, is magnified section of record line showing drop markings. For further explanation, see text.

Ratios of the lowering of edema formation were determined for four dilutions of one of the commercial extracts, ranging from 3×10^{-8} to 10^{-6}. The edema formation ratio for each concentration, when plotted against the logarithm of the concentration, resulted in a straight line. The upper limit of this line was fixed by the concentration which showed no effect on the edema formation rate and hence gave the ratio of 1. Concentrations between 10^{-2} and 10^{-4} gave edema formation rates of 0, while, at the other extreme, concentrations less than 10^{-9} had no effect on the rate of edema formation. The calibration curve is shown in figure 4.

By determining the edema ratio for a single concentration of an unknown sample and by referring to this line, it was possible to evaluate the unknown in terms of the substance used for obtaining the calibration curve. It was possible to determine directly concentrations of which the activity varied over three orders of magnitude. These determinations could be made with an accuracy sufficient to detect differences in concentrations of less than twofold.

With Kendall's extract, concentrations as low as 10^{-8} gave readily measurable effects. With one commercial extract, the measurable effect was pro-

[6] Dr. E. C. Kendall of the Mayo Foundation, Rochester, Minn. generously supplied us with samples of whole cortical extract and several crystalline compounds.

duced by less than one dog unit in 100 liters of the perfusion medium. Since 100 cc. of perfusion fluid is ample for an assay, it will be seen that a test can be made with approximately 0.001 of a dog unit.

When compared on the basis of grams of gland extracted Kendall's whole extract was found to be 2.5 times as effective in preventing edema as one commercial extract (X) and more than 300 times as effective as another extract (Y). The relative efficacy of these three extracts in preventing edema

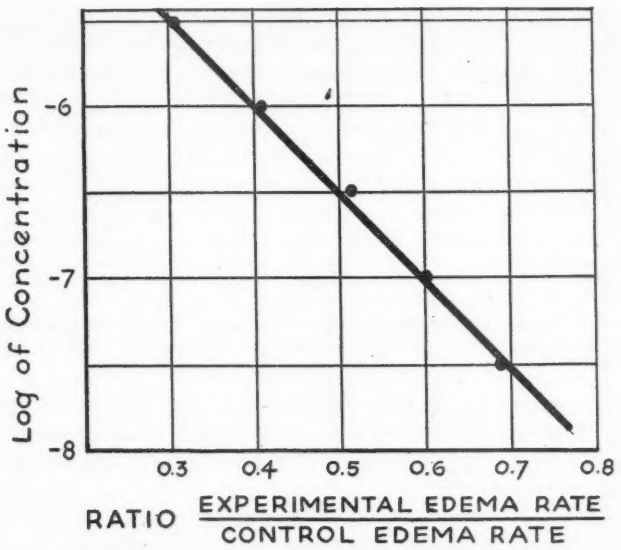

Fig. 4. Calibration curve. Ordinates represent the logarithm of concentration of cortical substance; abscissa, ratio of edema formation with cortical extract to edema produced without cortin.

formation is not in the same order as when the extracts are compared on the basis of dog units. Likewise, minimum detectable concentrations varied among the three compounds.

Effect of various steroids. Similar tests were made with a number of steroids and with strophanthin. The cortical steroids were 17-hydroxy-11-dehydrocorticosterone (Kendall's *E*), 11-dehydrocorticosterone (Kendall's *A*) prepared by Kendall, and synthetic 11-desoxycorticosterone. The other steroids were cholesterol, dehydrocholic acid and methyl testosterone. Strophanthin has been reported by Zwemer, Lowenstein and Pines (1940) to have an activity similar to that of adrenal-cortical extracts.

The relative effectiveness of the above compounds in reducing the edema formation rate are represented in the first column of table 1, which table is further amplified in the discussion. It is to be seen that Kendall's compound *A* was the most effective, a concentration of 0.05 mg. per cent reducing the edema formation rate to 0.4 of the normal. The next in order of efficacy was

synthetic desoxycorticosterone. Kendall's *E* was still less efficient, being only one tenth as effective as Kendall's *A*. The desoxycorticosterone used was a water-soluble preparation and, according to the manufacturer's statement, contained large amounts of urea and methyl urethane. The response therefore cannot be ascribed solely to the desoxycorticosterone, since the urea and urethane may exert an effect which is not known.

The other steroids were tested qualitatively since they could not be obtained in aqueous solutions of known concentration. Saturated solutions

TABLE I. EFFECT OF ADRENAL CORTICAL COMPOUNDS ON EDEMA FORMATION IN FROG'S HIND LIMBS

MATERIAL TESTED	EDEMA LOWERING EFFICACY	LIFE MAINTENANCE	SALT EFFECT	CARBOHYDRATE METABOLISM
Dehydrocorticosterone (Kendall's *A* compound)	10	+++	+++	+
Desoxycorticosterone	3	++++	++++	+
17-Hydroxy-11 dehydrocorticosterone (Kendall's *E* compound)	1	+	+	+++
Strophanthin	0.6	++	++	++
Cholesterol	0.1	no effect	no effect	no effect
Dehydrocholic acid	0.1	no effect	no effect	no effect
Methyl testosterone	no effect	±	++	

Numbers refer to relative efficacy in lowering edema formation. The number preceding the plus signs indicates the relative effectiveness of the substances on the physiologic phenomena heading the respective columns.

were used for adding to the control medium. Methyl testosterone gave no observable effect. Cholesterol and dehydrocholic acid gave significant effects but, according to their probable concentrations, were far less effective than the adrenal cortical steroids.

Strophanthin, at a concentration of 0.08 mg. per cent, produced a diminution of edema formation rate to about 0.6 of that in the control.

DISCUSSION

The principal feature involved in the method described in this paper is the use of a control perfusate the colloidal osmotic pressure of which is sufficiently low to produce a measurable rate of edema formation in the perfused legs of the frog.

The consistently obtained decrease in edema formation when adrenal cortical extracts are present in the perfusate is of considerable interest aside from its value as a method for assay. However, it is not possible at this stage of the investigation to conclude that the effect is due to a change in capillary permeability.

Eastman gelatin was used empirically as the colloidal constituent of the perfusate because, as an ash-free gelatin, it had been found serviceable in previous perfusion experiments dealing with the rôle of calcium on the intracellular cement of the capillary endothelium (Chambers and Zweifach, 1940). In the present experiments the ash-free factor was not essential since bacteriologic gelatin which contains mineral salts including calcium was found to be equally serviceable.

Other proteins were tried as the colloidal constituent of the perfusate. In the few preliminary experiments made they did not give results similar to those with gelatin, indicating that something in the nature of the colloidal constituent, possibly its dispersity, may have to be taken into account. The colloid which gave the most uniformly satisfactory results was the Eastman purified calfskin gelatin.

A few direct observations were made on the capillaries of the web of the foot during perfusions of the isolated hind limbs. A deficiency of perfusion through the capillaries was present irrespective of whether the perfusate contained or did not contain the extracts. These observations were made because of the possibility that the decrease in edema formation might be due to an action of the adrenal cortical extract in reducing the number of available vessels for the perfusion. Such an alteration would reduce the available surface area for outward diffusion of fluid with a resulting decrease in edema formation. Alterations of this nature might be expected in preparations with an intact nervous system. Dr. Zweifach of this laboratory (unpublished) has observed that the vasomotor activities of the microscopic muscular vessels, chiefly the arterioles, are enhanced by the injection into the circulation of adrenal cortical extracts. This would tend, under appropriate conditions, to re-route the perfusate. However, the preparations used in our experiments were not under central nervous control and the concentrations of cortical extracts were much lower than those used by Zweifach. Moreover, the rate of outflow in the perfusion experiments was always the same in the control and the experimental periods. Since the pressure is kept constant the uniform rate of flow indicates that there is no change in hydrodynamic resistance in the perfused vascular bed. However, this does not completely exclude the possibility of re-routing since two alternative routes may vary in their overall permeability and still possess the same gross resistance to flow.

Another possible explanation for the reduction of edema formation by adrenal cortical extracts is the diminished capacity of muscle tissue to bind water. Angerer and Angerer (1941) showed that the increase in weight of isolated frog muscle in hypotonic media was diminished when adrenal cortical extracts were added to the media. In our preparations free fluid collected in large pools under the skin and between the muscle bundles. This exudate had passed out of the circulatory system independent of the state of the muscle substance.

The reduction of capillary permeability by cortical extracts has been claimed by Menkin (1940) and by Freed and Lindner (1941). They observed a reduced escape of certain dyes from the circulation into regions of the skin altered by toxic agents. This was done by comparing the intensity in staining of skin areas injected with leukotoxin alone with that of skin injected with mixtures of the toxin and cortical substances subsequent to intravascular injection of trypan blue. In most cases the regions injected with the mixtures showed a less intense coloration, a result taken to indicate a decreased alteration of the capillary wall by the toxin.

If our results are taken to indicate a change in capillary permeability this

must be considered as a change from a condition of permeability regarded as a control standard to a condition of a lesser permeability. On the other hand, Menkin measures the efficacy of the cortical materials in counteracting the effect of agents which are claimed to increase the permeability above normal.

Freed and Lindner (1941) have stated that the effect of cortical extracts on capillary permeability is independent of their effects on other physiologic activities. In our experiments we have found that the several whole extracts which have been assayed in terms of life maintenance units showed great differences in their ability to lower the rate of edema formation when compared in dog units. It is therefore apparent that an adrenal cortical extract which assays high in terms of life-maintenance does not necessarily show a correspondingly high effect in mitigating edema formation. Likewise, in comparing a series of steroids, a corresponding variance was found. Table 1 shows that no parallel exists between the effects of these compounds on the lowering of edema formation, as measured by our method, and their effects on life maintenance, metabolism of carbohydrates, salts or water as compiled from generally accepted data.

CONCLUSIONS

Whole adrenal cortical extract from three sources, also cortical and related steroids, have been shown to reduce the rate of edema formation in perfused frog's legs. The effectiveness of cortical materials in reducing edema formation does not parallel other physiologic criteria of cortical activity.

A sensitive method for the bio-assay of adrenal cortical hormone is described which permits a rapid, quantitative detection in concentrations as low as 1/200,000 of a dog unit per cc.

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URINARY STEROIDS FROM BREAST CANCER PATIENTS[1]

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SEVERAL RECENT investigations concerning the relationship of steroids to human cancer have been directed toward the examination either of the excreta of cancerous individuals for the steroid content or of the excreta and tissues for carcinogenic potency. In one of the earlier studies Yolton and Rea (1940) reported that the estrogen and androgen excretion of two males with mammary carcinoma did not vary significantly from that of the normal male. Ross and Dorfman (1941) described similar findings in women with cancer of the breast. Werthesen and Pincus (1940) observed diminished androgen excretion in cancerous individuals, and Pincus and Graubard (1940) reported the estrogen excretion of cancerous females to be greater than normal. A later study by Pincus and Pearlman (1941) showed that cancerous women excreted less estrogen than non-cancerous women, and further that the ratio of non-ketonic to ketonic substances was greater in the former group. Pearlman (1942) found the 17-ketosteroid excretion to be decreased in cancer patients.

Utilizing isolation techniques Bloch and Sobotka (1938) demonstrated large amounts of cholesterol in the urine of cancerous patients, but later Sobotka, Bloch and Rosenbloom (1940) found this compound to be present in an amount not significantly different from that found in patients without malignancy. Pearlman (1942) established the presence of isoandrosterone and androstenone-17 in the urine of cancerous males, and isoandrosterone in that of cancerous females.

EXPERIMENTAL PROCEDURES

The selection of subjects for this investigation was limited to post-menopausal women with cancer of the breast. The urine was processed in three batches. The data concerning the patients and the extraction of the urine are given in table 1.

The urine of *Batch I* and about one-half of the urine of *Batch II* was hydrolyzed with hydrochloric acid and subsequently extracted with benzene. The method of Hirschmann (1940) was used. The remainder of *Batch II* and all of *Batch III* were processed by the simultaneous hydrolysis and extraction

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[1] From the dissertation presented to the Graduate School of the University of Colorado by Borroughs R. Hill in partial fulfillment of the requirements for the Ph.D. degree.

[2] Present address Converse Memorial Laboratory, Harvard University.

procedure of Dingemanse, Borchardt and Laqueur (1937), as described by Callow, Callow, Emmens and Stroud (1939), which uses carbon tetrachloride as a solvent.

Each separate batch was divided into acid, phenolic and neutral fractions by the extraction of a benzene solution of the urine concentrates with aqueous

TABLE 1. RESULTS OF FRACTIONATION OF THE THREE BATCHES OF URINE

BATCH	NUMBER OF PATIENTS	AGE OF PATIENTS	TREATMENT OF PATIENTS	VOLUME OF URINE	TOTAL EXTRACT	NEUTRAL FRACTION
		yr.		l.	gm.	gm.
I	6	52 to 70	Operation with recurrence in two. X-ray in one.	117.0	26.64	4.94
II	6	59 to 70	Operation with recurrence in two. X-ray in three.	57.7	9.61	1.59
III	8	49 to 70	Operation with recurrence in two. X-ray in four.	103.5	9.78	2.39

sodium bicarbonate, sodium carbonate and sodium hydroxide (cf. Callow, *et al.*, 1939).

Table 1 gives the quantity of the total concentrates and of the neutral fractions obtained from each batch of urine.

The isolation procedures were the same for the three lots of material and are described in detail for *Batch I* only. Any variations therefrom in the processing of *Batches II* and *III* are noted in the later descriptions. A summary of the fractions and compounds obtained from the neutral fraction of *Batch I* is given in table 2.

Batch I

The ketones were separated from the non-ketones with the Girard-Sandulesco reagent T (1936). The ketonic fraction weighed 1.12 gm. and the non-ketonic 3.42 gm.

Ketonic fraction. When the ketonic fraction was dissolved in hot 75 per cent ethanol for treatment with digitonin, cooling caused the separation of a small amount of precipitate. Recrystallization from acetone yielded 3.8 mg. of colorless needles, m.p. 233–235° C., with decomposition. This substance did not form an acetate. No further attempts were made to establish its identity.

The treatment of the material soluble in 75 per cent ethanol with digitonin, and the decomposition of the digitonides with pyridine yielded 5.8 mg. of a red gum from which no crystals could be obtained.

The digitonin was separated from the unprecipitated compounds by extraction with ether to yield 1.059 gm. of non-precipitable material. An attempt was made at this point to accomplish further purification by chromatographic separation. When no crystalline products were obtained, the fractions were recombined and subjected to further chemical separation.

Following the method of Wolfe, Fieser and Friedgood (1941), 545 mg. of the ketones were treated with succinic anhydride to separate the carbinols from the non-carbinols. The non-alcoholic, ketonic fraction thus obtained weighed 220 mg., and the alcoholic ketones weighed 180 mg.

The latter fraction (3-α-hydroxy ketones) was dissolved in 5 cc. of carbon

TABLE 2. URINARY EXCRETION OF NEUTRAL SUBSTANCES AS FOUND IN THIS STUDY ON WOMEN WITH BREAST CANCER, COMPARED WITH PUBLISHED DATA ON NORMAL, OVARIECTOMIZED AND CANCEROUS INDIVIDUALS

FRACTION OR COMPOUND	WOMEN WITH BREAST CANCER			NORMAL WOMEN[1]	OVARIECTOMIZED WOMEN[2]	CANCER PATIENTS[3]
	BATCH					
	I	II	III			
	mg./l.	mg./l.	mg./l.	mg./l.	mg./l.	mg./l.
Ketones	9.6	7.1	8.7	10.0	10.2	
Non-ketones	29.2	17.8	13.4	8.0	17.6	18.4
Alcoholic ketones precip. by digitonin	0.05	0.09	0.10		0.26	0.09
Alcoholic non-ketones precip. by digitonin	0.5	0.3	0.7			
Alcoholic ketones not precip. by digitonin	1.5	2.0	2.4			4.5
Alcoholic non-ketones not precip. by digitonin	0.5	2.5	3.8			
Ketones not precip. by digitonin	9.0	6.9	+		9.2	7.2
Non-ketones not precip. by digitonin	24.7	17.5	+		14.9	
Non-alconolic ketones	1.9	3.3	3.1			
Non-alcoholic non-ketones	12.1	8.5	7.7			
Androsterone	0.08	0.1	+	1.3	0.87	
3-α-hydroxyetiocholanone-17	0.09	0.17	+	1.3	1.14	
Pregnanediol	—	0.10	0.12		0.11	
Cholesterol	0.30	0.26	0.45		0.52	
Urinary hydrocarbon	0.19	+	+			
Dehydroisandrosterone	—	—	—	0.02	0.11	
Isoandrosterone	—	—	—			0.08

+ indicates presence without procurement of a quantitative yield
— indicates that the compound was not found
[1] Callow, N. H. and R. K. Callow (1939)
[2] Hirschmann, H. (1940)
[3] Pearlman, W. H. (1942)

tetrachloride and passed through an 8 × 110 mm. column of activated alumina (Brockmann). Fractional elution was made with carbon tetrachloride and alcoholic carbon tetrachloride (Hirschmann, 1940). The eluate was cut into 14 fractions varying from 20 to 100 cc. in volume. Crystalline material was obtained from *fractions* 7, 8, 11 and 12. Attempts to obtain crystals from the other eluates were unsuccessful.

Androsterone. Fractions 7 and 8, above, yielded crystals whose mixed melting point was the same as that of the individual substances. They were combined and recrystallized from aqueous methanol to yield 9 mg. of androsterone, m.p. 179–180° C. A mixed melting point with an authentic specimen[3] (m.p. 177.5–179° C.) gave a melting point of 178–179.5° C.[4]

[3] Androsterone was obtained through the courtesy of Dr. K. Dobriner, Memorial Hospital, New York, and Dr. S. Lieberman, Harvard University.
[4] All melting points are uncorrected.

Analysis $C_{19}H_{30}O_2$: calculated C 78.57, H 10.41
found C 78.53, H 10.44

The acetate was prepared by the treatment of androsterone with acetic anhydride in pyridine at 100° C. for 30 minutes. The addition of water yielded the crystalline acetate, m.p. 164–166° C., after recrystallization from methanol. The mixed melting point with authentic androsterone acetate (m.p. 164–165.5° C.) was 164–166° C.

3-α-hydroxyetiocholanone-17. Fractions 11 and *12* from the chromatographic separation of this group yielded crystals the mixed melting point of which was the same as that of the individual fractions. They were combined and recrystallized from aqueous methanol to yield 10 mg. of 3-α-hydroxyetiocholanone-17, m.p. 138–139° C. The mixed melting point with an authentic specimen[5] (m.p. 138–139° C.) was 138–139° C.

Analysis $C_{19}H_{30}O_2$: calculated C 78.57, H 10.41
found C 78.41, H 10.34

The semicarbazone was prepared by treatment of the compound with semicarbazide acetate in alcohol and pyridine. After standing over-night at room temperature, the semicarbazone was precipitated with water, and was recrystallized from dilute ethanol and dioxane to yield colorless needles, m.p. 253–255° C. (decomposition). When mixed with the semicarbazone of an authentic specimen of 3-α-hydroxyetiocholanone-17 (m.p. 253–255° C.) there was no depression of the melting point.

The non-alcoholic ketones (220 mg.) were subjected to chromatographic separation with an 8×130 mm. column of activated alumina using carbon tetrachloride and alcoholic carbon tetrachloride for elution. When no crystalline compounds were obtained, the fractions were treated with semicarbazide acetate. Semicarbazones of a workable nature were obtained from the first fraction only. This eluate (64 mg.) was treated with approximately 60 mg. of semicarbazide acetate in 1.5 cc. of 80 per cent ethanol and 3 cc. of pyridine. The mixture was allowed to stand over-night at room temperature. The addition of 5 cc. of water caused the separation of a heavy, white precipitate. After thorough washing with water this material was reprecipitated three times from dilute ethanol to yield 54 mg. of a substance, m.p. 232–235° C. (decomposition). Further attempts to purify this substance did not raise the melting point. The entire amount was then extracted several times with small quantities of hot, 95 per cent ethanol. The portion which did not dissolve was recrystallized from dioxane to yield 6 mg. of needles, m.p. 268–270° C. (decomposition). Further recrystallization did not raise the melting point. The alcoholic extracts were combined and concentrated to about one-third their volume, approximately 3 cc., and a white, amorphous substance separated. Reprecipitation of this material from ethanol yielded 10.4 mg., m.p. 252–254° C. (decomposition). The mixed melting point of these two preparations was 241.5–245.5° C.

[5] 3-α-hydroxyetiocholanone-17 was obtained through the courtesy of Dr. L. F. Fieser, Harvard University.

Non-ketonic fraction (cholesterol). The non-ketones were further fractionated with digitonin. The precipitated digitonides were decomposed with pyridine, and the partly crystalline residue (56 mg.) was recrystallized from ethanol, methanol and petroleum ether to yield 35 mg. of cholesterol, m.p. 147–148° C. There was no depression of the melting point when this was mixed with an authentic specimen of cholesterol, m.p. 147° C. The acetate, prepared with acetic anhydride in pyridine, melted at 115–116.5° C. and when mixed with an authentic specimen of cholesterol acetate did not depress the melting point.

When the non-ketones were dissolved in alcohol for the digitonin precipitation, a small amount of material failed to go into solution. This was recrystallized three times from acetone to yield 13 mg. of a substance with a melting point of 60.5–61.5° C. An additional 9 mg., m.p. 49–51° C., were obtained from the mother liquors. This compound had a tendency to revert to a non-crystalline material when dried. The analysis is at best an approximation because of the lack of an adequate criterion of chemical purity.

Analysis $C_{28}H_{58}$: calculated C 85.19, H 14.81
found C 83.93, H 13.96

The melting point, solubility properties and analysis suggest the identity of this compound with the urinary hydrocarbon described by Marker (1939).

The fraction non-precipitable by digitonin (2.89 gm.) was separated into carbinols (60 mg.) and non-carbinols (1.42 gm.) by the formation of the half-succinates in the manner previously described. Chromatographic separation of the carbinol fraction failed to yield crystalline products. The non-carbinol fraction, however, yielded a precipitate, recrystallization of which gave 5 mg. of the hydrocarbon, m.p. 60.5–61.5° C., which did not depress the melting point when mixed with the hydrocarbon obtained earlier in the examination of the non-ketonic fraction.

Chromatographic separation of the remainder of the non-carbinols did not yield crystalline material.

Batch II

Table 2 contains a summary of the fractions and compounds obtained from the neutral fraction of *Batch II.*

During the processing of this batch, the non-alcoholic ketones (180 mg.) were adsorbed from carbon tetrachloride on a 10 × 100 mm. column of activated alumina. The adsorbate was eluted with carbon tetrachloride and alcoholic carbon tetrachloride in 7 fractions. During attempts to induce crystallization, the first fraction was dissolved in hot acetone. Cooling caused the separation of a small amount of crystalline material which, after three recrystallizations from hot acetone, yielded 2 mg. of white needles, m.p. 233–235° C. (decomposition). The melting point of this compound when mixed with the ketonic substance (m.p. 233–235° C.) from *Batch I* was not depressed. The compound from *Batch I* was found in the ketonic fraction before the separation of the carbinols from the non-carbinols, but it did not form an acetate. Its presence

in the ketonic, non-carbinol fraction of *Batch II* gives added support concerning the nature of its active groups. Treatment of the compound with semicarbazide acetate in alcohol and pyridine yielded a substance which melted at 233–235° C. (decomposition). The fact that the melting point of the derivative was the same as that of the original compound cast doubt on the formation of a semicarbazone, but the mixed melting point of the two was depressed to 214–220° C. (decomposition).

Pregnanediol. The non-ketonic fraction, non-precipitable by digitonin (146 mg.) was adsorbed from 5 cc. of carbon tetrachloride on a 8×200 mm. column of activated alumina. The adsorbate was removed by fractional elution with carbon tetrachloride and alcoholic carbon tetrachloride in 9 fractions of 25 to 100 cc. Each fraction was dissolved in acetone and allowed to stand in the refrigerator for several days. *Fractions* 6 and 7 formed white, amorphous precipitates which, after several recrystallizations from acetone and dilute ethanol, yielded crystalline products whose melting points were similar and in the range of 226–231° C., and whose admixture caused no depression of the melting point. They were combined and recrystallized from aqueous ethanol to yield 6 mg. of white needles, m.p. 229–231° C. The melting point when mixed with pregnanediol from pregnancy urine, prepared by the method of Astwood and Jones (1941), was not depressed. The melting point of this preparation as well as that of the material from the urines of our patients was lower than that of pure pregnanediol. Astwood and Jones recognized the fact that their method produced a compound which was not entirely pure, and which they suggested might be contaminated with allopregnanediol. The possibility exists that our product was a mixture of these two isomers.

Analysis $C_{21}H_{36}O_2$: calculated C 78.69, H 11.32
found C 78.56, H 11.45

A 1.6 mg. sample of this compound was treated with 0.12 cc. of acetic anhydride in 0.2 cc. of dry pyridine at 100° C. for 30 minutes. Dilution with water yielded a precipitate which was thoroughly washed with water and recrystallized from aqueous methanol to give 1.2 mg. of a compound, m.p. 148–149° C. There was no depression of the melting point when this was mixed with an acetate prepared in the same manner from pregnanediol of pregnancy urine (m.p. 146–149° C.). These findings for the melting point of the original compound and its acetyl derivative, together with the analytical values, further indicate the possibility that the product was a mixture of pregnanediol and its allo isomer. The small amount available precluded the possibility of complete purification.

Batch III

Fractionation was accomplished by the same methods used for *Batches I* and *II* with the exception that the carbinols were separated from the non-carbinols before digitonin precipitations were made. A summary of the frac-

tions and compounds obtained from the neutral fraction of *Batch III* is given in table 2.

The smaller amounts of androsterone and 3-α-hydroxyetiocholanone-17 found in this batch are explained by the fact that when their presence was established no further attempt was made to isolate and purify all of the material remaining in the various mother liquors.

DISCUSSION

A comparison of the excretion of neutral substances by post-menopausal women having cancer of the breast with the excretion by normal women, ovariectomized women and cancerous women as found by other workers is given in table 2.

The mere presence in the urine of the steroids herein reported does not appear to bear any relationship to the existence of malignancy. The same steroids together with dehydroisoandrosterone and androstenone-17 were isolated from the urine of ovariectomized women by Hirschmann (1940). Our findings are more comparable to his than to those of other investigators because of the existence in his patients of an artificial menopause. Pearlman (1942) reported the presence of isoandrosterone in the urine of cancerous females of unstated age, but it was not found in this study nor by Hirschmann (1940). Among the compounds found by us, androsterone and 3-α-hydroxy-etiocholanone-17 (Callow and Callow, 1939), pregnanediol (Venning and Browne, 1937) and cholesterol (Marker, *et al.*, 1938) have been isolated from the urine of normal women. The compound with a low melting point, designated urinary hydrocarbon by Marker (1939), has been found by him in both animal and human urine and, consequently, its presence here does not indicate a connection with the existence of malignancy.

After our work was completed Dobriner, Gordon, Rhoads, Lieberman and Fieser (1942) reported the examination of the steroids from the urine of 6 cancer patients, 1 female and 5 males. They reported the presence of androsterone in the urine of 3 of the 6 patients, and 3-α-hydroxyetiocholanone in the urine from all of the patients. In addition, they found a compound which melted at 232–234° C. in the urine of one cancer patient and in that of 3 patients with adrenal hyperplasia. We encountered a non-alcoholic ketone with approximately the same melting point.

A comparison of the amounts of various fractions and compounds we found with those found by other workers should not be accredited too much significance. The procedures used in different laboratories are not identical and are not designed as quantitative methods. However, work such as that of Pearlman (1942) and Dobriner, *et al.* (1942), in which the same workers have made simultaneous studies of the urine of normal persons and of those with cancer, should carry comparative significance. Dobriner, *et al.*, found the range of concentration of β-ketosteroids to be greater in cancer patients than in normal individuals, whereas Pearlman found the concentration was decreased in cancer patients. Our results with *Batch II*, the fractionation of

which we consider to be quantitatively the most reliable, were exactly the same as those of Pearlman for this fraction.

SUMMARY

The urine of post-menopausal women with cancer of the breast, or with metastatic growths from this origin, was examined for its steroid content. Androsterone, 3-α-hydroxyetiocholanone-17, an impure form of pregnanediol, cholesterol and a low melting hydrocarbon were isolated.

Comparison of these results with those of other workers who have examined the urine of ovariectomized women, of normal women, or of cancerous men and women shows that our findings do not constitute positive evidence that the compounds isolated bear a relationship to the existence of malignant growths.

The microanalyses reported in this paper were performed by Dr. E. W. D. Huffman of the Huffman Microanalytical Laboratories, Denver, Colorado. They were made possible by a grant from the research fund of the Graduate School of the University of Colorado.

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RELATION OF THE B VITAMINS TO OVARIAN FUNCTION DURING EXPERIMENTAL HYPERTHYROIDISM[1]

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IT HAS BEEN well established that a relationship exists between the thyroid gland and the reproductive organs. Reiss and Pereny (1928) found that the threshold response of castrated rats to follicular hormone was raised during hyperthyroidism, and Weichert and Boyd (1933) and van Horn (1933) produced diestrus in normal rats by feeding thyroid gland. Halpern and Hendryson (1935) studied the effect of dinitrophenol on the estrous cycle of rats, but found only a slight increase in the length of the cycle, which they interpreted as being due to the toxicity of the drug.

Varied interpretations have been given as to the mechanism by which thyroid feeding affects the estrous cycle, and the problem is complicated by the effect of the concomitant loss of weight, and by the effect of food intake on the estrous cycle (Drill and Sherwood, 1938; Mulinos *et al.*, 1939). It has been shown that the amount of B vitamins in the diet will greatly influence the weight and food intake of hyperthyroid rats and dogs (Drill, 1938; Drill and Sherwood, 1938; Drill and Overman, 1942; Drill and Shaffer, 1942). If the B vitamins are fed to hyperthyroid animals in sufficient quantity a loss of weight can be prevented (Drill and Shaffer, 1942), and some evidence has been presented to show that the administration of the B vitamins will prevent the appearance of diestrous in hyperthyroid rats (Drill and Sherwood, 1938). Further experiments will be reported in this paper on relationships between food intake, body weight, estrous cycle and ovarian histology of hyperthyroid rats fed varying amounts of the B vitamins.

METHODS

Female rats, averaging 200 gm. in weight, were fed a diet free of the B vitamins, consisting of: casein, 20; crisco, 10; salts, 4; cod liver oil (U.S.P.), 4; and corn starch, 62 parts. This stock diet was fed *ad libitum* and the amount eaten each day measured. The thyroid dosage, yeast supplement[2] and the amount of B vitamins supplied per day by each supplement are listed in table 1. The thyroid gland fed contained 0.21 per cent iodine. At the con-

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[2] The authors are indebted to Dr. C. N. Frey of the Fleischmann Laboratories for supplying the analyzed yeast and yeast concentrate; to Dr. R. T. Major of Merck and Co., for supplying the crystalline vitamin B_1 and to Dr. H. W. Rhodehamel of Eli Lilly and Company, for the desiccated thyroid gland.

TABLE 1. THYROID DOSAGE, YEAST SUPPLEMENTS AND AMOUNT OF B VITAMINS SUPPLIED TO RATS

GROUP NO.		NO. OF RATS	THYROID FED PER DAY	YEAST FED PER DAY	AMOUNT OF B VITAMINS FED PER DAY IN YEAST SUPPLEMENT			
					B_1	RIBOFLAVIN	B_6	Ca. PANTOTHENATE
			mg.	mg.	I.U.	μg.	μg.	μg.
1	Control rats	9	0	200	4.6	12	17	24– 30
2	Negative controls	9	100	200	4.6	12	17	24– 30
3	Test rats	15	100	1000[1]	50.0	60	85	120– 150
4	Test rats	12	100	1000[1]	200.0	230	200	1500–2000

[1] Yeast concentrates.

clusion of each experiment the animals were autopsied and weights of the pituitary glands and the ovaries were taken. The ovaries from at least half of the animals in each group were examined after serial sectioning.

RESULTS

Group 1. The control rats gained an average of 28 gm. in weight and maintained normal estrous cycles throughout the experiment. The normal control animals consumed an average of 10.5 gm. of food per day (fig. 1).

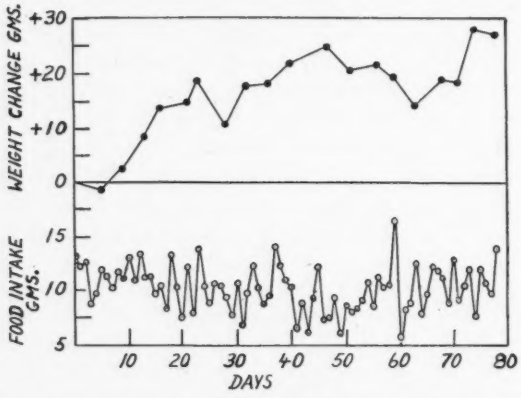

Fig. 1. Food intake and change in weight of the control rats (*group 1*), showing that the daily yeast supplement was adequate. These rats maintained normal estrous cycles throughout the experiment.

Group 2. The negative control rats, receiving 100 mg. of thyroid gland per day and only 200 mg. of yeast each day showed a marked and rapid loss of weight. The food intake of these animals remained essentially the same as that of the normal controls of *group 1* (fig. 2), no consistent decrease or increase in food intake being observed. These rats, which had normal estrous cycles prior to the start of thyroid feeding, exhibited only 2 or 3 estrous cycles after thyroid feeding was begun (fig. 4). Furthermore, after the 17th day of thyroid feeding none of the hyperthyroid rats in this group had an estrous

cycle. At the time the estrous cycles ceased the rats had lost 20 to 30 gm. in body weight, and by the 47th day of the experiment there was an average body weight loss of 54.3 gm. These rats were killed on *day 48* of the experiment.

Group 3. The test rats in this group received 1 gm. of a yeast concentrate per day, supplying 50 I.U. of vitamin B_1 per day (table 1). When thyroid feeding was started the rats began to lose weight and on the 23rd day had lost an average of 20.8 gm. in body weight (fig. 3). It was surmised from previ-

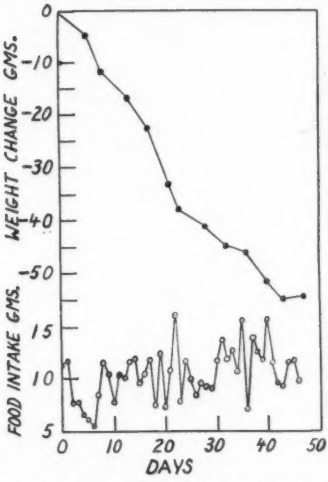

Fig. 2. (*left*) Food intake and weight change of hyperthyroid rats (*group 2*), receiving the same amount of yeast as indicated for the control rats in figure 1. The thyroid feeding produced a marked loss in weight, with no consistent change in food intake. The estrous cycles of these rats ceased after 10 to 17 days of thyroid feeding.

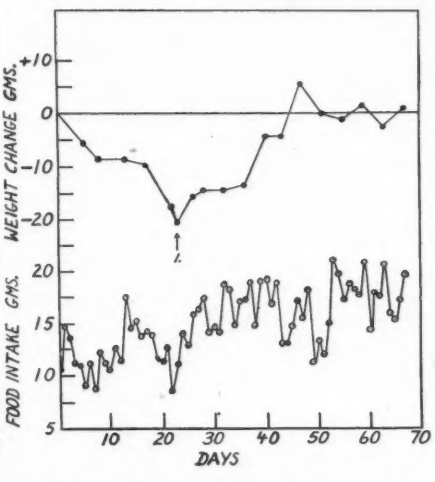

Fig. 3. (*right*) Food intake and weight change of hyperthyroid rats (*group 3*), receiving 1 gm. of a yeast concentrate per day. A slight initial increase in food intake is seen, followed by a return to normal, and a decline in body weight. It was surmised that the loss in weight was due to an insufficient supply of vitamin B_1 in the yeast concentrate; therefore at *point 1* all of the rats were injected with 500 μg. of vitamin B_1 per day. The food intake then increased to values above normal, the loss of weight was regained and maintained throughout the rest of the experiment. The rats also maintained normal estrous cycles throughout the 67 days of thyroid feeding.

ous work that the yeast concentrate did not supply sufficient vitamin B_1. Accordingly, on the 23rd day, the hyperthyroid rats in this group were injected daily with 500 micrograms of vitamin B_1. A rapid increase in food intake was then observed and the lost weight was rapidly regained. Food intake and body weight were then maintained by this regimen throughout the experiment, the rats consuming an average of 16.4 gm. of diet per day (fig. 3). The estrous cycles in this group remained essentially normal during the 67 days of thyroid feeding (fig. 4).

Group 4. The thyroid-fed rats in this group received, in addition, 1 gm. of yeast concentrate per day. This yeast concentrate was a more potent

source of the B vitamins than that supplied to *group* 3 (table 1) and this was reflected in the food intake of these hyperthyroid rats which was found to increase from a control level of 11.1 gm. per day to an average of 19.2 gm. per day. The rats increased in body weight (fig. 5) and the estrous cycles remained normal (fig. 4) until the time of autopsy, a period of 79 days. No additional injections of vitamin B_1 were given to this group as in *group* 3, since the yeast concentrate provided an adequate supply of vitamin B_1.

The weight of the ovaries from the control rats (*group* 1) and the hyperthyroid rats receiving a high B vitamin diet (*group* 4) were essentially normal

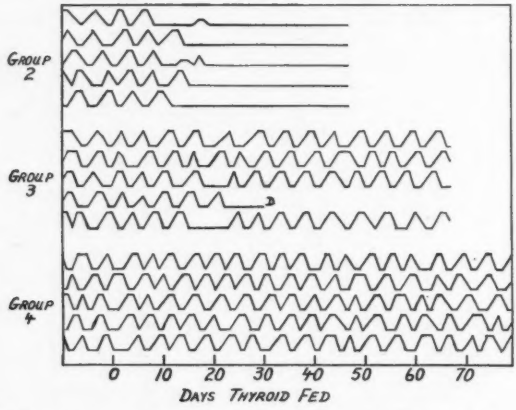

Fig. 4. Estrous cycles of hyperthyroid rats. Estrous cycles typical for 5 rats in each group are shown. D indicates the death of a rat. *Group* 2, the negative controls, receiving only a small amount of yeast each day, rapidly entered into continuous diestrus. *Groups* 3 and 4, receiving a high B vitamin diet, showed normal estrous cycles throughout the period of thyroid feeding.

(35.6 ± 3.9 mg. and 36.4 ± 4.3 mg.). However, the ovarian weights of the thyroid-fed control animals were significantly below normal, averaging 26.1 ± 4.8 mg. Correspondingly, the pituitary glands from the thyroid-fed control rats were significantly lighter in weight than the normal, being 8.3 ± 0.32 mg. as compared with 14.1 ± 0.29 mg. On the other hand, the weights of the pituitary gland from hyperthyroid rats receiving a high B vitamin diet were essentially normal, averaging 12.5 ± 0.33 mg., and were significantly heavier than the pituitaries from thyroid-fed controls.

The ovaries from a number of rats in each group were examined after serial sectioning. In view of the normal cyclic vaginal smears, a normal ovarian histology was anticipated in the untreated control animals (*group* 1), as well as in those rats that received thyroid, yeast concentrate and vitamin B_1 (*groups* 3 and 4). The usual maturing graafian follicles and large corpora lutea were observed in the ovaries of the animals in these groups. The ovaries from the negative controls (*group* 2) were reduced in size. In general, these organs exhibited a large amount of stroma, some sections being devoid of cor-

pora lutea as well as of follicles that had reached antrum formation. Corpora lutea of normal large size were observed in only two animals. Ovaries of other rats had corpora lutea, but they were estimated as no larger than one-half normal size, although the lutein cells appeared quite normal and vacuolation of the cells was seldom observed. Since medium sized vesicular follicles were observed, oögenesis can be considered to have been proceeding normally up to this stage, but atretic follicles of this size were also observed. Thus, since follicular maturation appears to proceed normally until the follicles are medium sized and atretic follicles and corpora lutea of this size are also present,

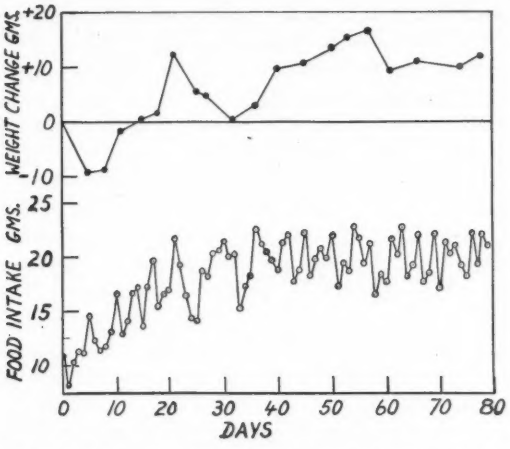

Fig. 5. Food intake and weight change of hyperthyroid rats (*group 4*), receiving 1 gm. of a yeast concentrate per day. When thyroid feeding was started the food intake showed a marked increase and the animals gained weight. The rats also maintained normal estrous cycles throughout the 79 days of thyroid feeding.

it is tempting to assume that the follicles never reach maturity, but are transformed into atretic corpora lutea. However, embedded ova were not observed and it is well known that atretic follicles may be seen in normal ovaries.

The loss of weight in the pituitary glands and the ovaries of the thyroid-fed controls would indicate that the functional capacity of the organs may be impaired, but ovarian histology suggested merely insufficient gonadotropic hormone secretions.

DISCUSSION

The results demonstrate that the feeding of a diet high in the B vitamins enables hyperthyroid rats to maintain normal estrous cycles and a normal ovarian histology and prevents a loss of weight of the pituitary gland. Before discussing the mechanism of this effect of a high B vitamin diet on estrous cycles during hyperthyroidism, it is pertinent to inquire how thyroid feeding produces diestrus. *a*) Evans and Simpson (1930) and van Horn (1931) reported that hyperthyroidism increased the gonad-stimulating potency of the pitui-

tary, and also that thyroidectomy lowered the gonadotropic potency of the pituitary (Evans and Simpson, 1930). Smith and Engle (1930) and van Horn (1931), however, could find no effect of thyroidectomy on pituitary potency. *b*) In addition to these effects on the pituitary gland, Reiss and Pereny (1928) and van Horn (1933) reported that the threshold for injected estrogen is raised during hyperthyroidism. Van Horn (1933) believed that the raised threshold to estrogen and the increased gonadotropic potency of the pituitary in hyperthyroidism was due to a physiologic castration effect on the pituitary, produced by the high basal metabolic rate, and resulting in increased estrogen elimination. Halpern and Hendryson (1935) tested this hypothesis by feeding dinitrophenol, but did not produce any permanent diestrus, whereas their thyroid-fed animals showed 1 or 2 normal cycles followed by continuous diestrus. *c*) Mulinos, *et al.* (5), believe that inanition and loss of weight are the primary causes of diestrus.

The negative controls in our experiments (*group 2*), entered continuous diestrus after 12 to 20 days of thyroid feeding (fig. 4), during which time they had lost 16 to 30 gm. in weight (fig. 2). Thus, the resulting diestrus could have been caused by any of the three reasons outlined above. However, the thyroid-fed rats in *group 3*, receiving a high B vitamin diet, lost an average of 20.8 gm. during the first part of the experiment (fig. 3), while still showing normal estrous cycles (fig. 4). This would tend to show that the loss of weight is not of primary importance as a factor in producing diestrus. The loss of weight in these rats was corrected when additional vitamin B_1 was supplied, and the normal estrous cycles were maintained throughout the rest of the experiment. The rats in *group 4* also maintained normal estrous cycles throughout the period of thyroid feeding. Our histologic studies of the ovaries of the rats in *group 2* suggest an insufficient secretion of gonadotropic hormone as the primary cause of the continued diestrous period in hyperthyroid rats. The rats in this group also showed a marked atrophy of the pituitary, which was prevented in the other hyperthyroid rats by feeding a diet rich in the B vitamins.

It has previously been demonstrated that the administration of sufficient B vitamins will enable hyperthyroid rats to regain any lost weight (Drill and Sherwood, 1938). This effect on body weight depends on food intake, for to combat the high metabolic rate the hyperthyroid rats must eat more food if body weight is to be maintained. If the food intake is limited, the administration of the B vitamins will not prevent a loss of weight (Drill and Sherwood, 1938). Similar findings were made in this report. The control rats consumed an average of 10.5 gm. of diet per day. However, the hyperthyroid rats in *group 2*, receiving the same amount of yeast as the control rats, did not show any consistent increase in food intake and rapidly lost weight. The hyperthyroid rats receiving the high B vitamin diet (*groups 3* and *4*) showed a marked increase in voluntary food consumption and maintained their body weight.

While we have shown that a high B vitamin diet will maintain normal estrous cycles in hyperthyroid rats, it is not yet clear whether this effect is

due to the increased food intake and secondary maintenance of body weight, or whether it is due to a more direct effect of the administered B vitamins on the pituitary gland.

SUMMARY

Hyperthyroid rats, receiving a synthetic diet and a small yeast supplement, rapidly lost weight when 100 mg. of thyroid gland was fed each day. The food intake remained essentially the same as that of the control rats. After 10 to 17 days of thyroid feeding all of these rats had entered continuous diestrus. By administering sufficient B vitamins to two groups of hyperthyroid rats the food intake was increased, the loss of weight was prevented and a slight gain in weight obtained, while normal estrous cycles were maintained throughout the 79 days of thyroid feeding.

The ovaries and the pituitary of the hyperthyroid rats receiving the small yeast supplement showed a loss of weight. The histologic picture of these ovaries suggested insufficient gonadotropic hormone secretion. The hyperthyroid rats receiving the high B vitamin diet maintained normal ovarian and pituitary weight, and showed a normal ovarian histology.

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MITOTIC ACTIVITY IN THE ANTERIOR HYPOPHYSIS OF FEMALE RATS OF DIFFERENT AGE GROUPS AND AT DIFFERENT PERIODS OF THE DAY[1]

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THE PRINCIPAL factor that induces mitotic activity in the hypophysis of sexually mature female rats is directly or indirectly related to the estrous cycle since a large number of mitoses are found only in the postovulatory phase of the cycle (Hunt, 1942). At this phase, however, there is a considerable individual variation indicating that other factors also influence the number of mitoses. The purpose of this paper is to consider some of these secondary factors. Among these, the age of the animal is perhaps the most important and there was some indication of this in the first series of glands examined but in order to obtain more complete data on mitotic activity in the hypophysis in different age groups an additional series of animals was studied.

The occurrence of a diurnal rhythm of mitotic activity in organs such as the kidney, submaxillary gland and epidermis has been stressed by others (Blumenfeld, 1938, 1942; Carleton, 1934). It was therefore considered of interest to determine whether there is a difference in the mitotic activity in the hypophysis at different periods of the day.

MATERIALS AND METHODS

Ninety-four rats ranging in age from 52 to 342 days were used in the study. The animals were divided into age groups of 40-day intervals, the average and range for which are given in table 1. The procedure of fixing, staining, and determining the area of the hypophysis examined has been described (Hunt, 1942).

Colchicine (Merck) was used in 9 of the 13 groups[2] of animals since it was considered that the accumulation of mitotic figures in the metaphase would make the counting more accurate especially in older animals in which the number of dividing cells would be few and scattered at any one time. The colchicine was injected 9 hours before autopsy and intraperitoneally in a solution prepared an hour or less before injection. Preliminary experiments

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[2] The results from five animals of *group 1*, seven of *group 2* and all of *group 3* and *10* were taken from a previous report (Hunt 1942).

showed that with 0.1 mg. or less of colchicine per 100 gm. of body weight many mitoses proceeded to anaphase and telophase. Since a dosage of 0.3 mg. per 100 gm. was in no case lethal and mitoses did not appear beyond the metaphase, it was considered as the optimum dosage.

The stage of the estrous cycle was determined by daily vaginal smears for several cycles. In each case it was known approximately when cornified

TABLE 1. MITOTIC ACTIVITY IN THE ANTERIOR HYPOPHYSIS OF GROUPS OF FEMALE RATS WITH AND WITHOUT COLCHICINE

GROUP	NUMBER OF ANIMALS	AGE, AVERAGE AND RANGE, DAYS	HOUR KILLED 24, MIDNIGHT	STAGE OF CYCLE WHEN KILLED[2]	MITOSES PER SQ. MM., MEAN ± S.E.
1[1]	6	58 (52–66)	11–12	III, IV	56.0 ± 4.7
2[1]	14	95 (80–111)	9–15	III, IV	31.0 ± 4.6
3[1]	3	337 (331–342)	11–12	III	1.5 ± 0.12
4	2	60	17	IV	275.0
		76	17	IV	457.0
5	6	96 (82–122)	17–18	III, IV	77.5 ± 12.0
6	8	148 (132–157)	16–17	III, IV	45.0 ± 6.5
6a	9	139 (130–143)	23–24	III, IV	49.0 ± 9.9
6b	10	134 (117–146)	7– 8	III, IV	12.9 ± 2.0
7	7	188 (169–206)	16–17	III, IV	32.0 ± 2.9
8	7	220 (209–242)	16–17	III, IV	15.0 ± 2.8
9	2	300 (283–318)	16–17	III	4.9 ± 0.4
10[1]	14	146 (66–223)	11–12	V	0.5 ± 0.2
11	6	113 (79–142)	16–17	V	3.2 ± 0.7

[1] The animals in these groups were not treated with colchicine. All others received 0.3 mg. colchicine per 100 gm. of body weight.

[2] The stages of the estrous cycle follow those used by Long and Evans in which *Stage III* (late cornified smear) and *Stage IV* (cornified and sparse leucocyte smear) are spoken of in this paper as the postovulatory phase. *Stage V* (leucocyte smear) is diestrus. The results from five animals of *group 1*, seven of *group 2* and all of *group 3* and 10 were taken from a previous report (Hunt, 1942).

cells first appeared with the round cells and animals were killed 18 to 36 hours later when the smear consisted either of cheesy masses of cornified cells or of cornified cells with a few leucocytes. For convenience these two stages, which Long and Evans (1922) classify as *Stages III* and *IV*, are called the postovulatory phase. Smears were made just before the administration of colchicine and just before killing the animal.

The area of each hypophysis examined for mitoses was approximately 5 sq. mm. of a coronal section 3 micra in thickness. Such an area includes about 50,000 cells with nuclei. There is no significant difference in mitotic activity in sections at different levels of the hypophysis and careful examination of one section through the widest portion of the gland gives an adequate determination of the mitotic activity.

RESULTS

The average number of mitotic figures in the hypophyses decreased as the animals became older (table 1). For 5 age groups, the animals of which received colchicine, the average mitotic activity is as follows:

GROUP	AGE, DAYS	MITOSES PER SQ. MM.
5	96	77.5
6	148	45
7	188	32
8	220	15
9	300	5

The action of colchicine is shown in the data in table 1 by comparing *group 5* with *group 2*. The animals were of approximately the same age and killed when in the same phase of the sex cycle. There was an average of 77 mitoses per sq. mm. in the hypophyses of the 6 animals in *group 5* receiving colchicine, and 31 per sq. mm. in the 14 animals in *group 2* (without colchicine). If, however, the results from two animals with exceptionally high counts are excluded from *group 2*, the average is 26 mitoses per sq. mm. Thus colchicine acting for 9 hours approximately tripled the mitotic count.

Using this standard of comparison, another group of 6 animals (*group 1*), not receiving colchicine with an average age of 58 days, may be included in the above series of age groups. The average mitotic activity of this group is 56 mitoses per sq. mm. which is about twice as great as that for the 95-day-age group.

The results of counts from two animals (*group 4*) which had been injected with colchicine were apparently exceptional. The mitotic counts in these two animals, 60 and 76 days of age, were 275 and 457 mitoses per sq. mm., respectively. These two cases and a few others with exceptionally high counts indicate that some animals are much more responsive to factors inducing mitosis than others.

The vaginal smears in all of the animals with high mitotic counts in the hypophysis were either in the late cornified or in the cornified and leucocyte stage. In some animals (not injected with colchicine) which had cornified cells and a few leucocytes in the smear there was an unusually high number of mitotic figures in the late phases of division indicating that the mitotic activity would soon have become reduced.

In contrast to the large number of mitotic figures in the hypophysis in the postovulatory phase the number in diestrus (*groups 10, 11*) was consistently low regardless of the age of the animal. As with the animals killed in the postovulatory phase, colchicine approximately tripled the mitotic count but even so it did not exceed 5 mitoses per sq. mm. and was usually less.

There was not only a rhythm of mitotic activity in the hypophysis related to the sexual cycle but also a diurnal rhythm. This is shown by comparing *groups 6*, *6a*, and *6b*, the animals of which were approximately the same age and killed in the same phase of the sexual cycle. The 8 animals of *group 6* were killed between 4 and 5 P.M.; the 9 animals of *group 6a*, between 11 P.M. and midnight; the 10 animals of *group 6b*, between 7 and 8 A.M. All had received colchicine. There was no significant difference between the counts for *group 6* and *6a* but in *group 6b*, the animals of which were killed between 7 and 8 A.M. the average number of mitoses was 12.9 per sq. mm.

as compared with 45 per sq. mm. for *group 6* and 49 per sq. mm. for *group 6a*.[3]

DISCUSSION

The decline in mitotic activity in the hypophysis as the animals become older is apparent. A similar decline may be shown if the weight is correlated with the mitotic activity since, in general, the mitotic activity decreases as the weight increases. This, however, is because the weight increases with advancing age and weight itself does not seem to be a factor that influences the variability as was shown in a group of 25 rats of approximately the same age (131–159 days), but varying in weight from 167 to 210 gm. In this group there was no correlation between weight and mitotic activity as there were as many mitoses in the hypophyses of the heavier animals, on the average, as in the lighter ones.

The decline may not be attributed to a slowing down of growth. The number of mitoses occurring during the postovulatory phase is too great to account for the increase in size and weight of the gland after sexual maturity (Hunt, 1942). According to the tables in Donaldson (1924) the weight of the hypophysis increases .0036 gm. while the body weight increases from 60 to 120 gm. (50 to 70–80 days). As the body weight increases another 60 gm. (80 to 150 days) the hypophysis again gains approximately .0036 gm. This constant rate of increase in weight of the hypophysis in relation to the body weight even during the period when there is a sharp decline in mitotic activity is considered as further evidence that the large number of mitoses in young, mature animals is not so much a growth phenomenon as one that takes care of replacement of cells. Other organs such as the adrenal and thyroid gland, in which degeneration and replacement of cells is an accepted fact, show an increase in weight with advancing age similar to that of the hypophysis but the percentage of dividing cells is only a small fraction of that in the hypophysis.

Although degeneration of the cells of the hypophysis of the guinea pig has been described by Kirkman (1937) and I have seen a similar phenomenon in the rat, attempts to determine quantitatively the number of degenerating cells have failed. One difficulty is that before fixing the gland, even without removing it from the base of the skull, there is likely to be some trauma affecting the cells so that they simulate degeneration after sectioning. This was noted in two cases in which it was known that the dissecting instruments accidentally punctured the glands slightly. In the sections a great many cells had pycnotic nuclei and densely staining cytoplasm.

There is indirect evidence that the postovulatory mitotic activity of the hypophysis is correlated with the physiologic changes that occur in the ovary. Although it has been stated that the maximum mitotic activity in the hypophysis occurred at about the time of ovulation (Hunt, 1942), it was

[3] Using Fisher's (1938) table for the significance of difference of means of small samples P = less than .01 in comparing *group 6* with *6b* and *6a* with *6b*. This means that there is far less than once in 100 times that the difference might be due to chance. Comparing *group 6* with *6a* $P = 0.8$ which indicates that the difference is not a significant one.

subsequently found that ovulation had probably occurred 12 to 24 hours earlier. This conclusion is based on the observation of ova in the tubes and by comparisons of the ovaries from 16 animals obtained in this series with the description of the growth of follicles by Boling, *et al.* (1941). If estrogen is responsible for the mitotic activity in the hypophysis, there is a 12 to 24-hour period between the time estrogen production reaches a maximum and the time it produces a maximum effect. This delay is comparable to that observed by Allen, Smith and Gardner (1937) who found a maximum stimulating effect on mitotic activity of the genital epithelia of the mouse 37 hours after injection of theelin.

Assuming that estrogen is the primary cause of mitotic activity in the hypophysis, the question arises why there is a decrease in mitotic activity as animals become older. While there is no evidence that there is any great quantitative or qualitative difference in the estrogen produced by animals 100 days and by those of 200 days of age there is a marked difference during this time in the mitotic activity of the hypophysis. It thus seems probable that as the cells of the hypophysis become older they undergo a physiologic change which inhibits mitosis.

This increasing refractoriness with advancing age of cells in the hypophysis to factors causing mitosis is not characteristic of all tissues. In the intestine, for instance, I have found as many mitoses in the glands of Lieberkühn of animals 500 days of age as in those of 50 days.

In addition to the conditions at certain phases of the estrous cycle and the age of the animal there are other factors that influence mitotic activity in the hypophysis. Others have found that mitotic counts varied with the time of day at which the animals were killed. Blumenfeld (1942) reported the lowest number of mitotic figures in the submaxillary gland of the rat between 2 and 4 A.M. Carleton (1934), on the other hand, found more mitoses at night in the epidermis of newborn mice. In the hypophysis there are fewer mitoses during the 9-hour period after midnight than at other periods of the day. Judging from preliminary observations this difference is not due to a variation in light since the mitotic activity was not modified by keeping the animals in darkness or by exposing them to artificial illumination. It may be that the decreased mitotic activity at night is due to the time of feeding since Blumenthal (1940) found that this was a factor that modified mitotic activity in the thyroid and adrenal gland of the guinea pig.

The use of colchicine has been criticized either because it inhibits or because it stimulates mitotic activity. In this series of animals the mitotic figures accumulated during a 9-hour period are approximately three times the number in uninjected animals. Probably the nearest approach to the determination of the time required for cell division was obtained by Lewis and Lewis (1917) who state that, 'From the present data 2 to 3 hours would be a fair estimate of the time involved.' One would expect, on this basis that if cell divisions were stopped in metaphase over a 9-hour period, then the number of mitotic figures would be tripled or quadrupled. This conforms very well with the observed results and it is concluded that the action of colchi-

cine, in the hypophysis at least, neither inhibits nor stimulates cell division but rather accumulates the mitotic figures that would normally occur.

SUMMARY

In different age groups of sexually mature female rats the postovulatory mitotic activity in the hypophysis was greatest in rats 50 to 80 days of age and gradually declined in the older age groups. In those animals 300 days or older the number of mitoses reached a low level and was no greater than that in young animals in diestrus. It was concluded that the decline in mitotic figures with advancing age is due to some change in the cells of the hypophysis that makes them increasingly refractory to factors that cause mitosis.

There were significantly fewer mitoses in the hypophysis during the 9-hour period after midnight than at other periods of the day.

The mitotic count was tripled in the hypophyses of animals receiving colchicine intraperitoneally 9 hours before death.

The author wishes to acknowledge his thanks to Dr. C. M. Goss for the animals used and for many helpful suggestions.

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ADRENAL FUNCTIONS IN THE OPOSSUM[1]

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SILVETTE and Britton's observation in 1936 that adrenalectomized opossums do not show a decrease in plasma sodium and chloride suggested a species difference in the function of the adrenal since in most mammals there is a lowering of the plasma sodium and chloride after adrenalectomy. A decrease in these ions occurs in the adrenalectomized opossum if it is deprived of food but not of water. This also occurs in the adrenalectomized opossum when it is lactating or suffering from diarrhea (Britton and Silvette, 1937). It appeared, therefore, that the opossum adrenal has little to do with sodium metabolism except perhaps during certain stresses.

The level of the electrolytes in the plasma and of the sugar in the blood have been determined in opossums which have survived adrenalectomy for a longer period of time than have any of these animals heretofore reported. No significant changes have been found either in the electrolytes or in the blood sugar. Our results indicate that the opossum adrenal does not play as important a rôle in electrolyte metabolism and in the maintenance of blood sugar as it does in the cat, dog and man.

METHODS

The 13 female opossums (*Didelphys virginiana*) used in this investigation were furnished by the Wistar Institute. They were kept in individual cages, being fed 150 gm. daily of the following diet recommended by the Institute: meat, 60 per cent (hog liver 2 parts to 1 part heart); vegetables, 10 per cent (any green vegetables); a dry powder, 30 per cent composed of 30 per cent soy bean meal, 25 per cent peanut meal, 25 per cent dried skim milk, 6 per cent oyster shells, 4 per cent alfalfa leaf meal, 4 per cent brewer's yeast, 4 per cent cod liver oil and 2 per cent iodized salt. The animals had free access to water at all times.

Adrenalectomy was performed in two stages through the lumbar approach. The second operation was not performed until complete healing of the first. Adrenal extract was injected for the first 3 days postoperatively. Adrenalectomized animals were kept in a room maintained at a nearly constant temperature (27 to 28° C.).

Blood was obtained by heart puncture, heparin being employed as anticoagulant. Glucose was determined by the Somogyi modification of the

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Shaffer-Hartman method (1937). Sodium was determined by the method of Butler and Tuthill (1931); potassium by Consolazio and Talbott's modification (1938) of the Shohl and Bennett method; and chloride by the modified Volhard-Harvey titration method (1932). The volume per cent of packed erythrocytes was determined by the method of Wintrobe (1933).

RESULTS

The animals survived for considerable periods of time after adrenalectomy. For 12 of the animals the days of survival were 5, 36, 38, 48, 52, 65, 69, 90, 123, 153, 288 and 439. One is still alive after 603 days. In two of the 12 (those surviving 52 and 288 days) death was due to heart puncture, while in a third (surviving 90 days) death appeared to be due to a perforated gastric ulcer. Four animals gained in weight after removal of the second adrenal, two of them 24 and 41 per cent, respectively. Adrenal tissue was found in none of the animals at autopsy.

Eventually all animals (except those dying from heart puncture) died with symptoms characteristic of adrenal insufficiency. These signs did not appear until a few days or weeks before death and consisted of loss of appetite, weakness in the hind legs and general reduction in activity.

TABLE 1. BLOOD CHANGES AFTER ADRENALECTOMY IN THE OPOSSUM

ANIMAL	DAYS ADRENALECTOMIZED	WEIGHT	Na	K	Cl	R.B.C.	GLUCOSE	REMARKS
		kg.	*m.eq./l.*	*m.eq./l.*	*m.eq./l.*	*vol. %*	*mg. %*	
1	Before	2.45	140.6	6.0	112.0	47		
	10	2.57	144.2	6.6	105.2	48		
	29	2.74	146.2	5.3	97.8	47		
	120	2.31	159.3	6.8	115.6	49		Survived 153 days
3	Before	2.92	143.0	4.8	101.0	50		
	10	3.06	150.5	5.2	114.0	44		
	29	3.27	143.5	5.1	104.6	51		
	107	3.30	159.6	6.7	116.5	49		
	223	3.53	142.3	5.9	115.0		82	
	272	3.59	140.2	5.6	111.6	50	80	
	288	3.64	153.0	7.1	109.0	49		Died at 288 days from heart puncture
5	Before	3.07	139.9	5.1	102.4	48		
	52		138.3	5.2	109.5	48		Died at 52 days from heart puncture
10	Before	3.10	144.3	4.1	103.0	47		
	45	3.10	168.4		125.7	47		
	176	3.20	141.1	6.3	102.0		83	
	224	2.76	136.0	5.6	110.5	40	73	
	262	3.20	189.0	13.3	99.0	45	93	
	269		153.0	8.5	105.0	48	75	Died at 439 days
12	Before	3.0	141.2	4.8		45		
	59		140.3	5.2	111.5	52		
	176	3.78	141.7				116	
	224	3.87	130.0	5.3	104.5	52	77	
	269	4.25	136.0	6.2	110.0	57	81	Alive at 603 days

Typical examples of the blood studies are shown in table 1. The electrolyte picture in the blood plasma showed little or no change from the normal. The plasma sodium was rarely below normal. Occasionally a high level was reached (animal 1, 159.3; 3, 159.6; 10, 168.4) but it did not persist. The plasma potassium, except in animal 10, remained at normal levels for the most part.

It was difficult to obtain blood which was satisfactory for glucose determinations because the animal often became excited. Determinations were made on heart blood obtained without an anesthetic. In table 2 values from

TABLE 2. GLUCOSE IN THE BLOOD OF NORMAL OPOSSUMS
(*mg. per cent*)

DATE	NO. 24	NO. 27	NO. 30	NO. 32
9/25	145[1]	113[1]	79	82
10/23	94	80	76	96
11/20	72	80	67	85

[1] Struggled violently.

normal animals are given. The values for blood sugar in the adrenalectomized animals were normal (table 1).

Adrenalectomy was followed by no significant change in the volume per cent of erythrocytes, except in one animal (animal 12) in which there was an increase from 45 to 57 volumes per cent. This animal also showed the greatest decrease in plasma sodium.

DISCUSSION

Britton (1931) adrenalectomized 9 opossums in one-stage operations by the anterior route. The longest period of survival was 33 days while 4 survived less than 3 days. Undoubtedly the trauma from double adrenalectomy was too great for an opossum to withstand. However, the results reported herein indicate that if the opossum is given adrenal extract for a short time postoperatively he survives for considerably longer periods than do cats or dogs similarly treated.

Silvette and Britton (1936) likewise reported short periods of survival (average 6 days) for 12 opossums after the single-stage operation. They found an increase in plasma sodium which at times was rather large. Their animals were prostrated or very weak. They also showed that adrenalectomized opossums were unable to excrete sodium and chloride readily. Our animals appeared normal until a few days before death when they became more sluggish and lost appetite.

In the discussion of their paper published in 1938 Silvette and Britton state 'Serum sodium and chloride (and also carbohydrate) levels in long-surviving (2–4 weeks) adrenalectomized opossums are reduced to the characteristic low levels reported for other species (Britton and Silvette, 1937).' But in the latter paper, to which they refer, a decrease in serum sodium

occurred only during lactation, diarrhea or deprivation of food alone (water allowed). If both food and water were withheld, the serum sodium was normal or above.

The failure of the opossum to show a decrease in plasma sodium under ordinary conditions would indicate the absence of a sodium factor. But the lowering of serum sodium following lactation or diarrhea in an adrenalectomized opossum suggests that in the normal animal the adrenal produces a sodium factor which operates in these emergencies.

In elasmobranchs it has been impossible to obtain evidence of production of a sodium-regulating factor in the interrenal gland (Hartman, *et al.*, 1940a). This is not surprising in an animal which lives in a saline medium.

It is noteworthy that plasma potassium in the adrenalectomized opossum remained within normal range. In other mammals a rise in potassium usually occurs within a few days after adrenalectomy. In only one instance, *i.e.*, once in animal 10, was the plasma potassium high. The sodium was also high. A week later both had decreased. It has been found in cats and dogs that a rise in potassium follows a marked state of adrenal insufficiency rather than a lack of any specific factor (Hartman, *et al.*, 1940b).

It is also noteworthy that many adrenalectomized opossums show no signs of insufficiency for considerable periods of time. If the opossum adrenal produces a sodium factor it appears to be effective only during an emergency.

Blood sugar remained normal in the adrenalectomized opossums in this series of experiments. Silvette and Britton observed a distinct decrease. The complicating factors of the after-effects of a major operation no doubt must be considered in their animals surviving a short time only.

No sugar determinations were made a short time before death. Granting that blood sugar may become low before death it is difficult to explain the maintenance of a normal blood-sugar level for weeks or months in the absence of an adrenal carbohydrate factor except that sufficient carbohydrate was ingested to do this.

In the body economy of the opossum the adrenal appears to be of less importance than in many other mammals.

SUMMARY

Thirteen female opossums were adrenalectomized. All but one survived more than one month.

Plasma sodium in the adrenalectomized animals was rarely below normal. Occasionally a high level was reached. Plasma potassium and blood-sugar levels remained normal.

The adrenalectomized opossum is likely to survive for weeks or months with little or no sign of insufficiency if not subjected to stress. This would indicate that the adrenal of the opossum is not so important in the body economy as that of other mammals.

We wish to thank Dr. E. J. Farris of Wistar Institute for opossums, and Dr. K. A. Brownell for the blood-sugar determinations.

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INACTIVATION OF ENDOGENOUS ANDROGENS BY THE LIVER IN RABBITS[1]

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PREVIOUS INVESTIGATIONS have demonstrated that several of the steroid gonadal hormones are inactivated by the liver. By experiments *in vitro* Zondek (1934) and Heller (1940) found that estradiol was inactivated on incubation with liver slices or minced liver. Israel, Meranze and Johnston (1937) observed that estrogens were inactivated after passing through a heart-lung-liver perfusion system but not after perfusion through a heart-lung system. Biskind and Mark (1939) and Biskind (1941) reported inactivation of estrone and estradiol and its benzoate ester after implantation of pellets of these materials into the normally situated spleen. Endogenous estrogens were shown by Golden and Severinghaus (1938) to be inactivated when ovaries were transplanted intramesenterically.

Similarly, some of the androgens have been found to be inactivated by the liver as demonstrated by the investigations of Biskind and Mark (1939) who reported that testosterone propionate pellets implanted into the normal spleen were ineffective in maintaining the secondary sex organs in the castrated rat. Biskind (1940) found that methyl testosterone does not exert its specific effects on the genital organs when implanted in pellet form into the normally situated spleen. Desoxycorticosterone was shown to have an androgenic effect by Hooker and Collins (1940). Burrill and Greene (1942) reported its acetate ester inactivated by the liver in rats when implanted intramesenterically in pellet form. However, androgens of adrenal origin were found to be unaffected by passage through the liver (Burrill and Greene, 1941). Burrill and Greene (1940) showed that when venous drainage of testicular implants passed through the liver endogenous androgens were ineffective in stimulating the castrate penis, prostate and seminal vesicle of the rat. Some androgenic effects were noted on these organs when testicular tissue was implanted subcutaneously. The experiments undertaken and reported here confirm some of the above investigations.

METHOD

Fifteen young adult male rabbits were completely prostatectomized and prostatic tissue implanted into the anterior chamber of the eyes as described

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by Benjamin, *et al.*, (1940). Approximately 3 weeks were allowed for vascularization and organization of the transplants before their areas were determined. A photographic method and a planimeter were used in making these determinations. Over a 10-day period six or more readings of the area were made and the average was expressed as 100 per cent, and after subsequent experimentation an increase or decrease in area was considered as greater or less than 100 per cent.

After the normal areas of the prostatic implants were determined the testes were then transplanted into the body cavity. Both gonads were brought through the inguinal canal into the perioneal cavity and the testes separated from their scrotal attachments by severing the gubernaculum. The tunica of the testes was then sutured to the muscularis of the jejunum with as little disturbance as possible to testicular tissue and to its normal blood supply. Approximately two weeks was allowed for establishment of vascular anastomosis with the mesenteric circulation. At intervals varying from 10 to 40 days after this operation a second operation was performed in which the spermatic cord (including the spermatic artery and vein) of each testis was ligated and severed. Moreover surgical removal of a block of the vasa deferentia did not alter the findings. This procedure was done in order to rule out any blood supply reaching the testes by the vasa deferentia. Thus, the mesenteric circulation constituted the only blood supply to the transplanted testes.

All transplants were photographed at least three times each week during the course of the experiment, and their calculated areas were plotted on coordinate paper. In addition, all prostatic implants and transplanted testes were excised and prepared for histologic study at the conclusion of the experiment.

OBSERVATIONS AND CONCLUSIONS

Transplantation of the testes to the wall of the intestine had no effect on the size of intraocular prostatic implants in 11 of the 15 animals studied when the normal blood supply had minimal interference. Two animals showed an average decrease of approximately 15 per cent in transplant areas and two animals showed an average increase of 12 and 19 per cent. Most of the animals exhibited only the normal fluctuations in transplant areas occurring in normal untreated animals (fig. 1, 2) which were described previously (Krichesky, *et al.*, 1941).

Following the second operation (interruption of the normal testicular circulation) prostatic implant areas in all but 3 animals responded by a decrease (fig. 1, 2) similar to the response described following castration (Krichesky, *et al.*, 1941). In some instances the decrease in area fell to less than 50 per cent of the preoperative level and remained at this low level for the duration of the experiment. Two animals exhibited no change in area after the second operation. In the first 12 days another animal showed a decrease in prostatic implant area to approximately 70 per cent of the preoperative level which was followed by an increase in area, reaching the normal level 45 days after interruption of the original testicular blood supply. At autopsy

these 3 animals were found to have adhesions of one or both testes to the body wall with visible vascular anastomosis through the adhesions. This suggested that some endogenous androgens were probably passing directly into the systemic circulation by way of the body wall and not through the portal circulation.

To establish the validity of the preceding conclusion, two animals exhibiting marked regression of the prostatic implants were subjected to a

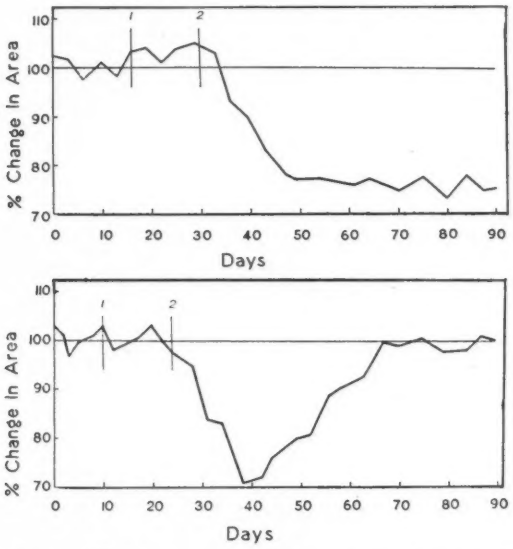

Fig. 1. (*upper*) Changes in area of intraocular prostatic implants in a male rabbit after transplantation of both testes to the jejunum, followed by interruption of the normal testicular blood supply; 1 indicates the time of transplantation of the testes and 2 indicates the time of severance of spermatic arteries and veins.

Fig. 2. (*lower*) Changes in area of intraocular prostatic implants in a male rabbit after transplantation of both testes to the jejunum, followed by interruption of the normal testicular blood supply. Secondary vascular anastomosis between one transplanted testis and the body wall had occurred; (1 and 2 as indicated in figure 1).

third operation; the transplanted testes with only mesenteric circulation were sutured to the body wall. In both of these animals prostatic implant areas increased, approaching the 100 per cent or preoperative level, in approximately 40 days after suturing the testes to the body wall. Adhesions with vascular anastomosis to the body wall were observed at autopsy. The above findings gave data which permitted the conclusion that some endogenous androgens reached the systemic circulation because the prostate implant areas in these two animals enlarged to the preoperative area.

Histologic study of the transplanted testes excised as long as 50 days after interruption of the normal testicular circulation revealed persistence of interstitial cells (fig. 3, 5). Seminiferous tubules also were present, but these were

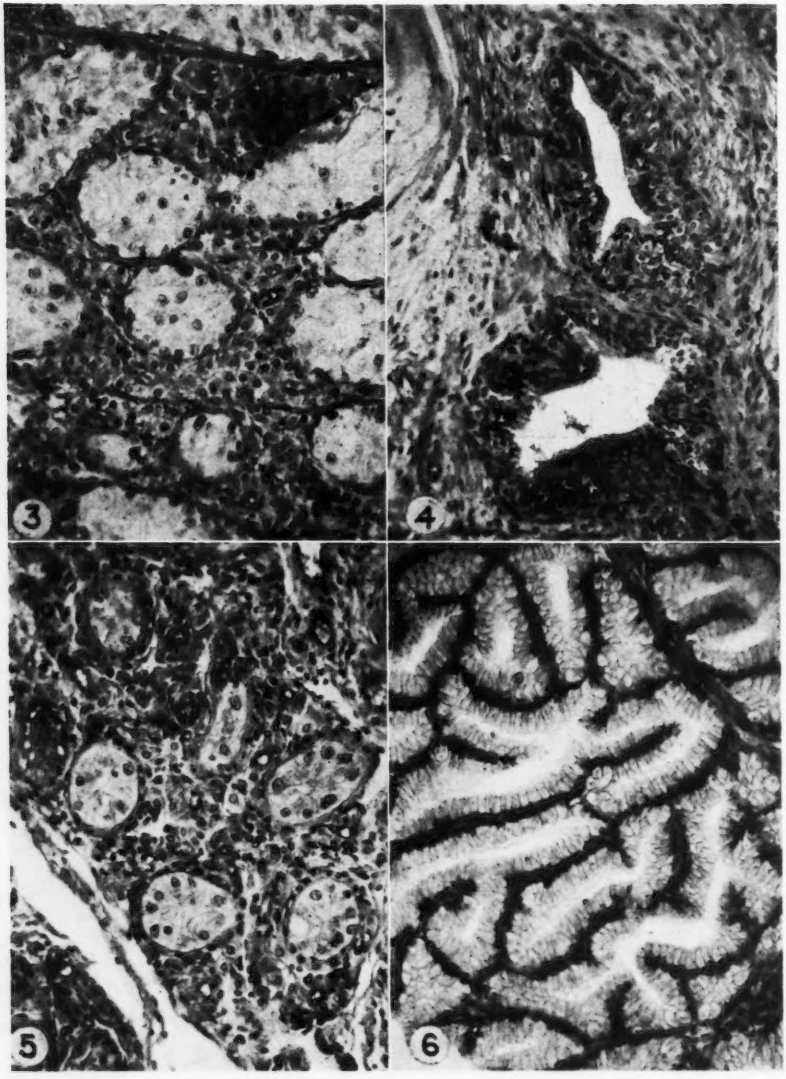

Fig. 3. Transplanted testis from the animal the prostatic area for which is shown in figure 1. There is a reduction in the size of the seminiferous tubules and a complete absence of spermatogenesis, but persistence of interstitial cells which appear hypertrophied. Delafield's hematoxylin and eosin; ×180.

Fig. 4. Intraocular prostatic implant the area of which is shown in figure 1. There is marked reduction in the height of the epithelium to a cuboidal and low columnar type and a complete absence of epithelial infoldings. Delafield's hematoxylin and eosin; ×180.

Fig. 5. Testis from the animal the prostatic area for which is shown in figure 2. This testis had vascular anastomosis with the body wall. Histology similar to testis shown in figure 3. Delafield's hematoxylin and eosin; ×180.

reduced in diameter and consisted only of Sertoli cells. No evidence of spermatogenesis was observed. Stained sections showed an increase in connective tissue, round cell infiltration, and increase in the size and number of the interstitial cells (fig. 3, 5). This strongly suggested but did not prove a continued production of male hormone by the transplanted testes.

Prostatic implants removed 40 days after interruption of normal testicular circulation (when their areas had decreased to 60 per cent of their preoperative level) exhibited characteristic castrate cellular structure (fig. 4). The glandular epithelium was not only markedly reduced in amount but it had also changed to a cuboidal and a low columnar type. There was a complete absence of epithelial infoldings. In those transplants from animals in which a secondary circulatory anastomosis between transplanted testes and body wall had occurred, the histologic structure was indistinguishable from that of implants from normal animals (Krichesky, *et al.*, 1941). The implants consisted mostly of glandular epithelium with a narrow core of fibromuscular tissue (fig. 6). The gland cells were of a tall columnar type, with granular cytoplasm and basal nuclei. Numerous epithelial infoldings were evident. The apparently normal structure of these implants suggested that a sufficiently high level of androgens existed in the systemic circulation to maintain (or restore) the prostatic tissue.

DISCUSSION

The ineffectiveness of transplanted testes in maintaining prostatic tissue when venous drainage from the testes transplants was passed through the portal circulation was clearly indicated by the changes in areas of intraocular prostatic implants. These findings, in agreement with those of Burrill and Greene (1940), suggested an inactivation of endogenous androgens by the liver.

The presence of interstitial cells in the transplanted testes indicated a continued production of male hormone. Further evidence of male hormone production was offered by the restoration of prostatic implants to normal size and structure in those animals which developed secondary vascular anastomosis to the body wall either spontaneously or experimentally. These findings were in agreement with those of Jeffries (1931) and others who reported that cryptorchid testes continue to produce androgens in nearly normal amounts for a considerable period.

It should be pointed out that only a part of the total testicular hormone produced might be sufficient to maintain prostatic tissue in its normal condition. Vascular anastomosis between testes transplants and body wall involved only one testicle in 3 animals, two spontaneously and one experimentally. Thus only part of the venous drainage from one testicle passed to the systemic circulation. In all 3 of these animals the areas and the histologic structure of the prostatic implants returned to the normal condition.

Fig. 6. Intraocular prostatic implant the area of which is shown in figure 2. There is a tall columnar epithelium, basal nuclei, and numerous epithelial infoldings (cf. fig. 4). Delafield's hematoxylin and eosin; ×180.

SUMMARY

Whole testes of the rabbit were transplanted intramesenterically with minimal trauma or disturbance to their normal circulation. After establishment of vascular anastomosis with mesenteric circulation, the original circulation via the spermatic arteries and veins was severed, resulting in venous drainage only through the portal circulation. Passage of endogenous androgens through the portal system and the liver failed to maintain prostatic implants. A later secondary vascular anastomosis to the body wall stimulated growth of the prostatic implants and restored their normal histologic structure. Evidence was obtained indicating that only a portion of the total androgen production by the testes was sufficient to maintain transplanted prostatic tissue.

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EFFECTS IN THE DOG OF INFUSION OF ADRENAL CORTICAL EXTRACT ON EXERCISE TOLERANCE, BLOOD CONSTITUENTS AND ADRENAL CORTEX[1]

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THE ASTHENIA and susceptibility to fatigue manifest in adrenal insufficiency, and the relief afforded by administration of adrenal cortical extract, have led to the suggestion that cortical extract might increase exercise tolerance and postpone fatigue in the normal animal. In the normal rat, however, very large doses of cortical extract have been found to cause cortical atrophy (Ingle, Higgins and Kendall, 1938); this property was found peculiar to those components with an oxygen on C_{11} (Wells and Kendall, 1940), although Selye (1940) claims to have found desoxycorticosterone effective. The experiments described herewith were undertaken to determine whether repeated intravenous infusion of whole cortical extract in the normal dog would affect *a*) the exercise tolerance, *b*) the level of blood constituents related to adrenal cortical activity, or *c*) the histologic structure and bulk of the adrenal cortex.

METHODS

Six dogs, weighing 16 to 30 pounds, were used for the study of exercise tolerance and constituents of the blood in relation to adrenal cortical activity. Determinations were made of serum sodium, serum potassium, blood nonprotein nitrogen, and blood sugar in the normal dog before and one hour after exercise, using venous blood drawn 20 to 24 hours after feeding. Each day of the experiment the dogs were made to rest until the resting heart and respiratory rates could be determined, then they were made to run in a treadmill, each at as fast a rate and for as long a time as he could be persuaded. The rates varied from 7.7 to 13.5 miles per hour, the times from 10 to 30 minutes, but the running time and rate were constant for each dog. Immediately after running the heart and respiratory rates were determined, and recorded repeatedly thereafter until the rates had reached the former resting level. By this means the exercise tolerance and the willingness of each animal to run were determined together with the acceleration of the heart and respiration as the result of exercise, and the cardio-respiratory recovery-time after exercise.

After several days of observation of the normal animals, one control dog was infused daily with 250 cc. of normal saline and the 5 experimental dogs

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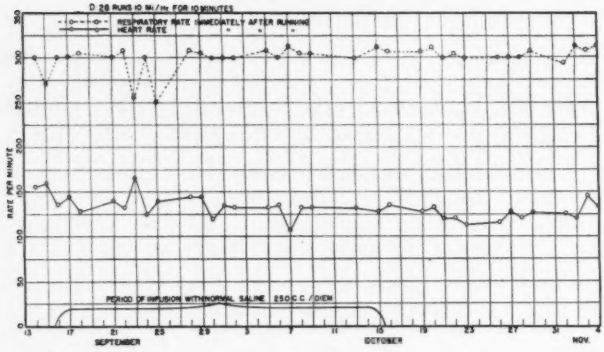

Fig. 1. Effect of daily infusion of normal saline on the exercise tolerance and post-exercise heart and respiratory rates in the normal dog.

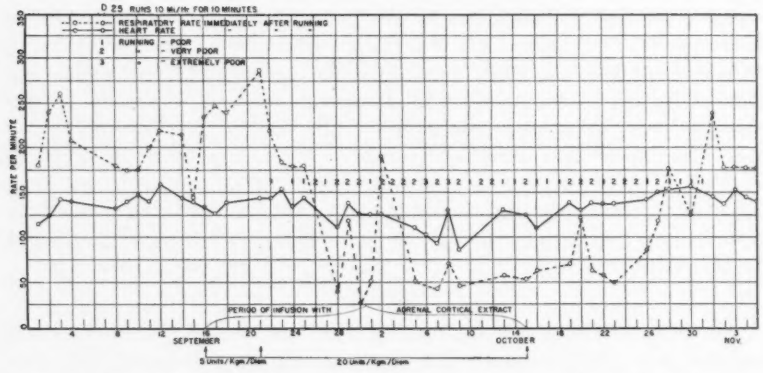

Fig. 2. Effect of daily infusion of adrenal cortical extract on the exercise tolerance and post-exercise heart and respiratory rates, in the normal dog. The poor running during the period of infusion probably accounts for the low post-exercise respiratory rate.

with a solution of adrenal cortical extract[2] containing 5 dog units per kg. of body weight in 250 cc. of normal saline at such a rate as to require 1.5 to 2 hours for complete infusion. Later in the day the dogs were exercised and the response to exercise recorded. Periodically the blood chemical studies were repeated on blood drawn before infusion. In 2 dogs the 5 units/kg. dose was infused daily for one week, and in 3 dogs daily for three weeks; thereupon the dosage for all was increased to 20 units/kg./day and the infusion continued daily for 3 additional weeks. Exercise and blood chemical studies were continued for 3 weeks after the cessation of infusions.

RESULTS

Effects on Exercise Tolerance and Recovery Time

On a daily dose of 5 units/kg. of cortical extract, the animals ran normally, but, after several days of injections they breathed more rapidly at the

[2] The adrenal cortical extract, supplied by the Connaught Laboratories, Toronto, Canada, contained 0.033 mg. per dog unit of tricresol as a preservative.

TABLE 1. EFFECT OF REPEATED INFUSION OF ADRENAL CORTICAL EXTRACT ON BLOOD SUGAR, BEFORE AND AFTER EXERCISE, IN THE DOG

DATE, 1942	D-25—MALE BLOOD SUGAR, MG. PER CENT		D-26—FEMALE BLOOD SUGAR, MG. PER CENT	
	BEFORE EXERCISE	AFTER EXERCISE	BEFORE EXERCISE	AFTER EXERCISE
Normal, before infusion period				
7/30	60	68	68	99
8/ 6	62	77	62	70
11	69	73	71	69
13	66	68	66	68
18	65	69	69	70
25	74	71	74	74
Period of infusion, 9/16–10/15/42				
9/21	69	62	60	73
28	75	79	70	75
10/ 6	81	79	84	86
7	74	69	72	69
14	75	80	83	79
Post-infusion period				
10/16	102	82	95	82
17	81	86	84	86
18	81	85	82	77
19	79	81	81	81
20	90	86	92	93
21	76	79	81	83
22	70	79	86	79

end of exercise than they had after exercise without cortical extract. There was no change in the recovery time.

On 20 units/kg./day a gradual reluctance to run developed in 4 of the 5 treated dogs. They would stop running and sit down, or require coaxing to continue; in one animal the speed was reduced from 7.7 to 5 miles per hour. In 3 of the dogs the respiratory rate immediately after exercise was higher than the normal post-exercise rate; in 2 it was lower. One of these latter, a female, ran as well but no better than normally; the other, a male, refused to run at all well, which may account for the decrease of the post-exercise respiratory rate (fig. 2). The periods for recovery from exercise were at times somewhat prolonged in 3 of the dogs. The striking feature of the experiment was the reluctance to run, rather than a respiratory and cardiac distress after exercise.

After the daily infusions were stopped, the unwillingness to run ceased promptly in 2 of the dogs, but only after 2 weeks in the other 2 animals.

In figures 1 and 2 are shown typical effects as produced by infusions of normal saline in the control dog and of cortical extract in an experimental animal.

Effects on Blood Chemistry

Glucose. Table 1 shows the following typical results. During the normal control period, exercise usually increased the blood-sugar level slightly.

During the infusion period the resting blood-sugar level was higher than the normal, but exercise produced the characteristic response. Early in the post-infusion period, the resting level was much higher than the normal, and exercise produced a sharp decrease; the resting level fell somewhat thereafter and exercise produced little change in blood-sugar level.

The sharp decrease in blood sugar induced by exercise so soon after withdrawal of cortical extract might be an indication of adrenal cortical damage; if so, it must have been repaired easily. The sustained rise in the fasting blood-sugar level might have one of a variety of causes.

Nonprotein nitrogen. The blood N.P.N. level was not disturbed during the infusion period, varying from 33 to 41 mg. per cent. Two days after infusion was stopped, the level rose to 50 and 51 mg. per cent; the following day and for 2 weeks it was at normal level. Evidently there was no serious disturbance of the blood nonprotein nitrogen.

Sodium and potassium. The serum sodium and potassium levels were not disturbed during the infusion period nor at any time thereafter.

Effects on Adrenal Weight and Cortical Activity

Three other dogs were given a daily infusion of whole adrenal cortical extract, 20 units/kg., for 3 weeks, without daily exercise, and were killed by the injection of 'Dial' on the day following the last infusion. The adrenal glands were removed, fixed in formalin, trimmed clean of fat and connective tissue, and weighed. The ratios of total adrenal weight to body weight in these dogs were .00009, .00013, and .00018. The ratios for 15 normal dogs killed in the laboratory varied from .00008 to .00014, with a mean of .00010. The ratios for 3 of the experimental dogs killed 2 months after infusion was stopped were .00010, .00010, and .00011, respectively. From this it may be concluded that infusion of adrenal cortical extract in the quantities used does not reduce the weight of the adrenal glands.

Histologic sections were made of the glands removed from the 3 dogs which were killed immediately after a daily infusion of cortical extract for a period of 3 weeks. Neither with hematoxylin and eosin, nor with Romeis Sudan lipid stain was any pathologic or atrophic change seen in the adrenal cortical tissue.

DISCUSSION

Experiments involving the effects of adrenal cortical extracts on muscular activity have ordinarily been made with rats. In the experiments of Herron, *et al.* (1934) the gastrocnemius was excited directly by electric shocks, and the output of work measured until exhaustion or sloughing occurred. Ingle (1938) reported no difference in the work performance between normal animals and normal animals given cortin. Such experiments, while recording accurately the capacity of muscle to do work, do not demonstrate the animals' willingness to work.

Dorrance, Thorn, Tyler and Katzin (1942) measured work performance of rats by inducing them to run in a revolving cage in which they might be

subjected to sub-atmospheric oxygen tension. They found that adrenal cortical extract neither increased nor decreased the work performance in rats rendered relatively anoxic in this apparatus, but that desoxycorticosterone acetate increased the work performance by about 6 per cent. This latter they did not consider significant. Selye and Dosne (1942) on the other hand, found that desoxycorticosterone acetate reduced the exercise tolerance in the rat forced to run in a revolving cage under normal atmospheric conditions.

The performance of our dogs confirms the contention of Long (1942) that 'up to the present time there has not been any demonstration that the administration of cortical hormones will improve the working capacity of the muscles of normal animals.'

Repeated infusion of adrenal cortical extract might be expected to cause atrophic changes in the adrenals, but the effective dose in the rat is many times that given to our dogs. Ingle (1938) demonstrated that muscular exercise tends to enlarge the adrenal gland in the rat, but that the enlargement may be prevented by administration of cortical extracts. In our experiments the adrenals of the exercised dogs were no larger than those of animals not given exercise, and the adrenals of the latter were no smaller than those of control animals. This suggests that the exercise was not sufficiently arduous to cause enlargement of the adrenals, and that the cortical extract, in the dosage given, did not cause atrophy of the glands in the dog. The reduction in exercise tolerance may, however, indicate a functional suppression.

SUMMARY

Slow intravenous infusion of whole adrenal cortical extract in the dog in dosages as large as 20 dog units per kg. daily for 3 weeks, produced no increase in exercise tolerance. During the period of infusion 4 of 5 dogs demonstrated great unwillingness to run in a treadmill. This unwillingness was not accompanied by any noticeable cardio-respiratory embarrassment and the reluctance gradually passed off, after the infusions were stopped.

Neither during nor after the period of infusion was there any disturbance of blood nonprotein nitrogen, serum sodium or serum potassium. There was a tendency to a slight rise in the blood-sugar level. Exercise lowered the blood sugar for a brief period after infusions were stopped. The cortical extract produced no cytologic change or atrophy in the adrenal cortex.

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ESTIMATION OF THE SIZE OF COMB ON LIVE FOWL

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WHETHER the size of comb is employed as a quantitative measure of the activity of androgenic hormone, or as an indicator of breeding capacity, its use for such purposes depends upon an accurate estimation of comb size. As early as 1932 Koch pointed out that 'at present writing, units used by different laboratories cannot be interpreted in terms of each other. There are so many factors involved in comb growth response and in accurate measurement thereof that considerable investigative work should be done before we attempt to adopt an international standard.' However, no adequate study has been reported which evaluates the different types of measurements of comb size that are commonly used.

The comb of the fowl has been employed by many investigators as an organ with which to assay the potency of androgenic hormones. In studies of reproduction, Blyth (1928) found the comb to be an indicator of the onset of spermatogenesis in young males. Among young Barred Plymouth Rock cockerels, Schnetzler (1938) observed that those with large combs were most likely to mate and to fertilize eggs.

This study was therefore undertaken to determine the relative accuracy of various techniques employed by investigators for estimating comb size.

Review of Techniques Reported

The weight of a comb can be assumed to measure adequately its size, being subject only to errors in the proportion of the comb removed from the head. The use of weight has the advantage of allowing for variations in the thickness of the comb which cannot be accounted for by linear measurements. Where possible it therefore seems desirable to indicate the size of the comb by recording its weight. This procedure has been employed by several investigators engaged in critical experiments in which the birds could be killed. However, the nature of some experiments involving a study of comb size makes this type of measurement impossible, and necessitates the use of a different procedure. The weight of the comb is presumably the most accurate quantitative measurement of its response to external or internal stimuli so that a factor for comb size which is closely correlated with the weight of the comb should be an accurate and desirable measurement.

Various criteria of comb development, other than weight, have been em-

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ployed, but thus far there has been little evidence that these measurements are closely correlated with comb weight, or with each other. Until such relationships have been determined there is little basis for comparison of the results obtained in different laboratories. No attempt has been made in this study to trace the origin and development of the techniques.

The length has been employed to measure comb size in the live fowl and has the advantage of extreme simplicity.

The length and height of the comb, considered separately, measures two of the three dimensions in which growth occurs, but pairs of figures rather than a single figure must be considered in making comparisons. The sum of the length and height has been used to eliminate this disadvantage. Two measurements combined in a single figure facilitate comparisons. Asmundson, Gunn and Klose (1934) have stated that this method gives no accurate indication of differences in weight. A comb measurement of 127 mm. (L + Ht.) weighed 12.1 gm., which was 41 times greater than the weight of a comb measuring 37 mm. One-half of the sum of comb length and height has been used in some studies, apparently in order to reduce the final figure to one that is intermediate between the two dimensions of the comb.

The product of comb length and comb height has been employed to stress comb size in terms of surface area rather than linear dimesions. Freud, de Fremery and Laqueur (1932) pointed out that this is a better measurement of the size of the comb than the sum of the length and height because the comb resembles a parallelogram in shape and the product of the sides of a parallelogram is more characteristic of its area than is the sum of its two sides.

The square root of the product of comb length and height has been employed, apparently to measure the size of the comb in terms of the length of one side of a square having comparable area. This figure may aid in visualizing actual changes in dimensions of the comb, and if this transformation increases the accuracy with which comparisons can be made, its use is undoubtedly justified even though the calculation of the square root is required.

A relatively complicated method has been used by several investigators in which the area of the comb is determined from measurements of a photograph, or from a projected image, by means of a planimeter. Evidence that the determination of the area by such means is subject to considerable error has been furnished by Gallagher and Koch (1935) who concluded that the direct measurements were more accurate and were more easily and rapidly obtained. It would appear from their report that the disadvantages of the planimetric method, or of modifications of it, are so great that its general use is not justified, and hence it has not been employed in this study.

The weight of life-size drawings was used by Morató-Manaro and Albrieux (1939) who 'impressed' the outline of the comb on photographic paper. This outline was then redrawn on a paper of uniform thickness, cut out and weighed. By comparison of the weights obtained from the same comb before and after the experimental period, the percentage increase or decrease in size was calculated. Aside from the fact that the method is time consuming, the results show only the relative size of the comb and give little indication of the actual size.

Measurements of comb size have been made by means of photoelectric cells by Gradstein (1935). The method is rather complicated and involves special equipment which is not practical for field work. The results compared rather closely with those obtained by the planimetric method. Photoelectric measurements, in common with any method utilizing silhouettes of the comb, have the additional disadvantage of difficulty in establishing accurately the base line for the comb, particularly when the comb is large and the base line is not straight.

Greenwood and Crew (1927) measured the volume of the comb by making an accurate impression of the comb with dental wax. From these impressions permanent plaster casts were made. The comb volume of the subject could then be determined. Although this method presumably gives a reliable estimate of comb size, and can be used on living birds, the complexity of the technique makes it impractical for routine use.

The present study was undertaken to determine which of five different

measures of comb size *a*) length, *b*) height, *c*) the sum of the length and height, *d*) the product of length and height and *e*) the square root of the product of length and height, is most closely correlated with the actual weight of the comb.

EXPERIMENTAL PROCEDURE

Males from three different strains of White Leghorns were studied. Cockerels from one strain (*Group I*), hatched on March 4, 1941, were reared in a colony house with free range until September 24 at which time the 20 birds were placed in a pen 10×14 feet; here they remained until November 13, at which time they were killed and experimental data obtained. The birds were 249 days of age at death, and had developed combs of considerable size.

The birds in *Groups II* and *III*, obtained from a local hatchery as 1-day-old sexed chicks, were autopsied at 70 days of age after they had been subjected to different periods of daily illumination throughout their lives. Those in *Group III* received 14 hours of artificial light daily; those in *Group II* received three 1-hour periods daily; otherwise treatment was the same. Both groups were brooded under electric brooders in pens 10 by 14 feet, so constructed that sunlight could not enter. At 8 weeks of age they were placed in laying cages where they remained until autopsy at 10 weeks of age. Before the birds were killed the combs were measured so that measurements of the comb could be compared with the weight of the comb obtained immediately thereafter. The greatest anteroposterior length of the comb was recorded and the height was measured as the perpendicular distance from the base of the comb to the highest point directly above. Measurements were made with calipers graduated in millimeters.

RESULTS

In order to determine the most suitable criterion of comb size, other than weight, correlations between the weight of the comb and its size as determined by five different methods were computed using data from the groups of birds reared at Cornell and from a group of Leghorn males reared in Kentucky and reported by Buckner, Insko and Martin (1932). The results of these computations are shown in table 1.

There is a remarkable uniformity in the relationship of the various measurements to the weight of the comb and the coefficients of correlation are uniformly high. Regardless of whether comb weight is correlated with the length, the height, the sum of the length and height, the product of the length and height, or with the square root of the product of length and height, the coefficients of correlation are highly significant ($P = <0.01$). This would indicate that all of these methods of measurement are useful for estimating comb size, but that the product of length and height in each group gave the highest coefficient of correlation with comb weight, whereas the sum of the length and height and the square root of the product of length and height gave slightly lower correlation coefficients. The coefficients of

correlation between the weight of comb and either the length or the height were somewhat lower.

The usual procedure for determining whether coefficients of correlation are significantly different is to apply Fisher's z_r technique. This method cannot be used in this case because of the peculiar manner in which all of the linear measurements of the comb are correlated with the comb weight and with each other. Recently, however, a method for testing the significance of differences between coefficients of correlation has been developed (Hotelling, 1940; Baten, 1941) and has been applied in testing the significance of the differences between the coefficients of correlation for length and weight, and for height and weight of the comb in each group. The method cannot be

TABLE 1. CORRELATION COEFFICIENTS BETWEEN COMB WEIGHT AND OTHER MEASUREMENTS OF THE SIZE OF COMBS ON WHITE LEGHORN MALES

GROUP	BIRDS	AGE	COEFFICIENT OF CORRELATION (R) BETWEEN WEIGHT COMB AND ITS				
	NO.	DAYS	LENGTH	HEIGHT	L+HT.	L×HT.	$\sqrt{L \times HT.}$
1	20	249	0.790	0.825	0.845	0.854	0.846
2	46	70	0.923	0.898	0.935	0.939	0.933
3	44	70	0.918	0.902	0.947	0.956	0.948
4[1]	18	168	0.932	0.936	0.952	0.962	0.952

[1] Computed from data presented by Buckner, Insko and Martin (1932)

used to test the significance of differences between coefficients of correlation when both depend upon the same measurements.

It will be noted in table 1 that the differences between the coefficients of correlation for comb length with weight and for height with weight are relatively small in each group of birds. Application of Hotelling's test shows that these differences are not significant. Neither length nor height shows a consistently higher correlation coefficient with weight, and these may therefore be considered of equal value if a single measurement of the comb is to be employed.

It is obvious, then, that both the height and length of the comb should be determined in experiments of this nature. Moreover, of all of the combinations of measurements mentioned (L+Ht., L×Ht., $\sqrt{L \times Ht.}$) that of the product of length and height is most closely related to comb weight. On this basis it is recommended in studies concerning the size of comb, especially when the weight of the comb cannot be readily obtained, that the product of the comb length and height be utilized as the measurement of comb size.

SUMMARY

Comparisons have been made between different linear measurements of the size of combs on 128 White Leghorn cockerels. The data from each of four groups of birds reared in four different environments, show that the product of the length and height of the comb gave higher coefficients of correlation with comb weight than did the measurements of length, height, the

sum of the length and height or the square root of the product of the length and the height. The coefficients of correlation for the sum of the length and height and for the square root of the product of the length and height were not significantly lower than those for the product of the length and height.

In order to facilitate comparison of results obtained in different laboratories, it is suggested that the measure of comb size which has the highest coefficient of correlation with comb weight be used when the comb weight is not available.

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EFFECT OF AUTOLYSIS ON THE DIFFERENTIAL SOLUBILITY OF THE PRINCIPLES OF THE POSTERIOR LOBE OF THE PITUITARY BODY[1,2]

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THE ORIGINAL purpose of this investigation was to determine whether the marked disproportion between the pressor and the oxytocic principles in the posterior lobe of the pituitary glands of whales described by Geiling (1935) and associates (1936), was due to a differential autolysis of the principles of the gland before removal or to a disproportion peculiar to the species. Geiling and associates found the pressor activity of these glands to be 100 per cent of the standard, but the oxytocic content varied from 10 to 40 per cent of the standard. However, Oldham (1938) showed that a similar disproportion appeared in the armadillo under conditions that seem to exclude differential autolysis as the only explanation. In the latter work the glands were removed from the armadillos immediately after death. On the other hand, glands removed from the small white whale (porpoise) assayed 100 per cent of the standard for both hormones (Geiling and Coon). Therefore, the question still remained whether the previously reported departure from the 1:1 ratio between the pressor and the oxytocic hormones of the hypophysis of the large whales, which could only be removed from 10 to 24 hours after death, could be ascribed to autolysis, to species difference, or to both. However, numerous difficulties were encountered during the progress of this work, and the original purpose was abandoned in favor of the intensive study on the effect of solvents on powders prepared from the posterior lobes of autolyzed glands.

Numerous studies have been made on the effect of solvents on the hormones from the neural lobe of the hypophysis. Only those pertinent to this study are referred to here. Baudoin (1913) reported that cold alcohol was a poor solvent, but that boiling alcohol would dissolve the active principles. Crawford (1920) found that the pressor principle was insoluble in absolute alcohol at 20° C., but soluble in acidified alcohol. Kamm (1926) and his coworkers showed that acetone would dissolve the oxytocic principle four

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[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Chicago.

[2] The essential portions of this dissertation were presented before the American Society for Pharmacology and Experimental Therapeutics at the meetings in Baltimore, Maryland, 1938, and in Toronto, Canada, 1939.

times as readily as the pressor principle from the glands left for some hours at room temperature. They claimed that this increased solubility was due to the acidity produced by the glands on standing. In the work reported here, a study was made of the effect of varying periods of gland incubation on the differential solubility of the pressor and oxytocic principles in mixtures of warm solvents.

EXPERIMENTAL PROCEDURES

Three types of material were incubated, *a*) beef heads with the pituitary gland *in situ*, *b*) individual glands and *c*) posterior pituitary powder with and without fresh anterior and posterior lobe tissue or tissue juices added. These were incubated at 22 or at 37° C., for periods ranging from 1 to 48 hours.

a) Beef heads were incubated from 6 to 48 hours at 37° C., to simulate as far as possible the conditions of whale pituitary gland before its removal. The blubber of the whale is an effective insulating material, maintaining the temperature of the body while in the water for 10 hours or more after death. Forty-two beef heads in 11 different lots were obtained directly from the killing floor of an abattoir. They were taken to the laboratory within one hour after the death of the animals and incubated in a constant temperature room for varying lengths of time. After incubation, the pituitaries were dissected out and frozen with carbon dioxide snow. The posterior lobes were then separated and placed in C.P. acetone and made into powders from which solutions were prepared according to the directions in the U.S.P. XI. Glands in heads incubated 48 hours were too decomposed to permit differentiation of the lobes and could not be used.

b) The difficulties encountered in incubating whole beef heads led to a study under similar conditions of freshly removed beef pituitary glands. The moisture content of the glands was maintained by placing them in suitable containers. After incubation they were frozen with carbon dioxide snow, the lobes separated and placed in acetone to obtain a powder.

c) A series of experiments were carried out with 5 to 10 cc. volumes of 0.25 per cent acetic acid extracts of U.S.P. posterior lobe powder by incubating these extracts with fresh anterior or posterior lobe tissue or juices. These juices were prepared by macerating one gland in 5 cc. of saline. Two drops of this tissue extract were used for each experiment.

Quantities of 100 mg. of the different powders so prepared were extracted with acetone, methyl,[3] ethyl, propyl, isopropyl, butyl, amyl and isoamyl alcohols by means of the micro-soxhlet extractor. Mixtures of acetone with the various alcohols were also used. The extractor held 10 cc. of solvent before siphoning over and the flask contained 20 to 22 cc. of solvent, depending on the relative quantities of the solvents present. When using mixtures of acetone and alcohol the amount of powder was kept constant so that the relative quantities of these two solvents condensed in the extractor would be uniform. The apparatus was set on a thermostatically controlled hot plate at

[3] At the time this work was done the author was not aware of British Patent No. 334,898, which advocates the use of acidified methyl alcohol for extraction of the oxytocic principle.

60° C. Anhydrous materials were used and anhydrous conditions were maintained throughout the experiments by means of calcium chloride. The last condition was essential, as extraction over prolonged periods of time permitted the extracting menstruum to absorb enough moisture to destroy the activity of solute and residue.

The various extracts of the posterior lobe reported in this paper were assayed for both pressor and oxytocic potencies. The pressor activity was measured by the manometric blood pressure method on cats under sodium phenobarbital anesthesia. The oxytocic activity of the extracts was assayed by the chicken method recently described by Coon (1939). In most cases the results of the latter were verified by the official (U.S.P. XI) guinea pig uterus method.

RESULTS

Most of the powders made from glands incubated at 37° C. in the intact beef head for 24 hours or less, assayed 90 to 100 per cent of the standard. Such powders, with occasional exception, contained equal quantities of the oxytocic and pressor principles. The hormone content from the 11 different lots is shown in table 1.

TABLE 1. HORMONAL CONTENT OF POSTERIOR LOBES OF PITUITARY GLANDS INCUBATED AT 37° C. IN THE INTACT BEEF HEAD

NUMBER OF HEADS	HOURS OF INCUBATION	PERCENTAGE OF U.S.P. STANDARD	
		PRESSOR	OXYTOCIC
4	6	95–100	95–100
4	12	95–100	95–100
6	18	95–100	95–100
2	20	90–95	90–95
4	20	90–95	60–65
4	24	95–100	95–100
10	24	90–95	90–95
2	24	70–75	50–55
2	24	20–25	15–20
2	24	10[1]	15–20
2	48	[1] Too decomposed to assay	

In a few instances these powders contained depressor properties. Abel (1919; 1920) and his associates, Dudley (1923), Dale and Dudley (1921), Jackson and Mills (1919), Hanke and Koessler (1920) and Schlapp (1925) have observed this depressor effect and have demonstrated that it is due to histamine and not to the active principles of the pituitary gland. When the powder from the glands incubated for 12 hours or more was extracted in a microsoxhlet extractor for 30 minutes with absolute ethyl alcohol to remove the depressor property the oxytocic activity of the extracted powder was found to be only 50 to 60 per cent of the standard, while the pressor activity remained unchanged. The remainder of the oxytocic activity was in the alcoholic extract. The glands from heads that were incubated for less than 12 hours did not show this separation of the oxytocic fraction so markedly.

In the series of experiments in which freshly removed glands with their

membranes intact were incubated in covered glass containers, essentially the same results were obtained as with glands incubated *in situ*. Glands incubated at room temperature gave more consistent results than those incubated at 37° C. Tables 2 and 3 give the data for this group of experiments.

The results of the experiments with the U.S.P. powder incubated with fresh anterior or posterior lobe tissue or juices were variable. In the majority of cases the loss of the activity was slight. In 30 such experiments inactivation was observed in 8, in which the inactivation ranged from 10 to 90 per cent. The destructive effect of the fresh posterior lobe and anterior lobe tissues and tissue juices was essentially the same. This irregularity is not unique, since Kamm (*et al.*, 1926) found similar occasional losses in activity in powders prepared by the official method. At the present time no adequate explanation can be given for this occasional decrease in potency of the active principles and for the variations in the ratio between them. Heller (1937) has shown that adsorption may be a factor.

Table 2. Whole pituitary glands incubated at 37° to 38° C.

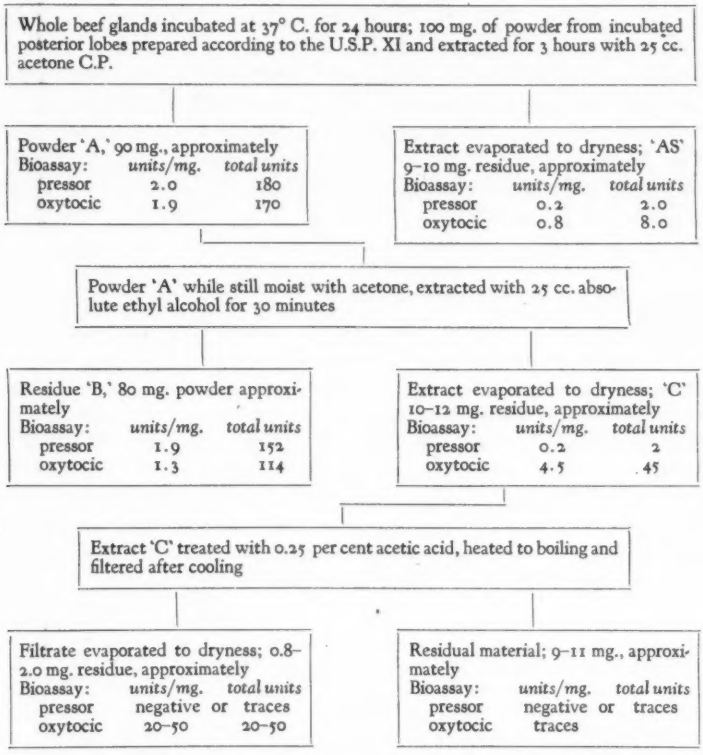
NUMBER OF EXPERIMENTS	HOURS OF INCUBATION	PERCENTAGE OF U.S.P. STANDARD, XI	
		PRESSOR	OXYTOCIC
1	1	100	100
1	2	100	100
1	4	95–100	95–100
2	6	95–100	95–100
1	12	95–100	95–100
1	20	90–95	90–95
1	20	90–95	55–60
5	24	90–100	90–100
1	24	85–90	85–90
1	24	50	50
1	24	20–30	10–20
1	24	20–30	20–30

Table 3. Whole pituitary glands incubated at 20° to 22° C.

NUMBER OF EXPERIMENTS	HOURS OF INCUBATION	PERCENTAGE OF U.S.P. STANDARD	
		PRESSOR	OXYTOCIC
1	1	100	100
1	2	100	100
1	4	100	100
2	20	95–100	95–100
2	24	95–100	95–100
1	24	95–100	60–65

In using the various solvents it was noted that there was greater solubility of the active principles of the powder prepared from incubated glands than those of the powder prepared from fresh glands. In the extraction with hot acetone the hormones of the incubated powder showed greater solubility than did those of the standard powder. The oxytocic hormone was removed about four times as readily as the pressor hormone.

The flow sheet indicates the manner in which the various glands were processed, and the bioassay figures for one typical experiment.


Whole beef glands incubated at 37° C. for 24 hours; 100 mg. of powder from incubated posterior lobes prepared according to the U.S.P. XI and extracted for 3 hours with 25 cc. acetone C.P.
Powder 'A,' 90 mg., approximately
Bioassay: units/mg. total units
pressor 2.0 180
oxytocic 1.9 170
Extract evaporated to dryness; 'AS' 9–10 mg. residue, approximately
Bioassay: units/mg. total units
pressor 0.2 2.0
oxytocic 0.8 8.0
Powder 'A' while still moist with acetone, extracted with 25 cc. absolute ethyl alcohol for 30 minutes
Residue 'B,' 80 mg. powder approximately
Bioassay: units/mg. total units
pressor 1.9 152
oxytocic 1.3 114
Extract evaporated to dryness; 'C' 10–12 mg. residue, approximately
Bioassay: units/mg. total units
pressor 0.2 2
oxytocic 4.5 45
Extract 'C' treated with 0.25 per cent acetic acid, heated to boiling and filtered after cooling
Filtrate evaporated to dryness; 0.8–2.0 mg. residue, approximately
Bioassay: units/mg. total units
pressor negative or traces
oxytocic 20–50 20–50
Residual material; 9–11 mg., approximately
Bioassay: units/mg. total units
pressor negative or traces
oxytocic traces

Powders prepared from fresh pituitary glands obtained from the abattoir did not yield an alcohol-soluble oxytocic fraction after extraction for 30 minutes with absolute alcohol, as did powder 'A' from the incubated glands. According to Kamm and his collaborators when U.S.P. posterior pituitary powder is extracted in a soxhlet apparatus for 6 hours with absolute ethyl alcohol, approximately 10 per cent of the oxytocic hormone is removed. However, with autolyzed powder, in our experiments, extraction for as long as 70 to 100 hours with a 1:10 ratio of acetone to methyl alcohol gave a residual powder assaying 85 per cent pressor and approximately 5 per cent oxytocic activity. This is the most significant finding of this investigation.

A similar experiment was performed with sperm whale powder prepared by the official method and originally assaying 100 per cent pressor and 10 per cent oxytocic activity. In this case, soxhlet extraction was carried out for 65 hours with a 1:20 ratio of acetone to ethyl alcohol, and gave a residual powder containing 20 per cent pressor activity and no oxytocic activity.

The oxytocic hormone was more readily soluble in hot methyl alcohol than in hot ethyl alcohol. The greater losses in activity obtained on prolonged extraction with ethyl alcohol were no doubt due to the higher boiling point of this solvent. The other alcohols mentioned were found unsuitable because of their higher boiling points.

The foregoing experiments would seem, therefore, to comprise additional data on the differential solubilities of the pressor and oxytocic principles of the posterior pituitary. The facts presented, notably the greater solubility of the two fractions when derived from autolyzed glands, point rather to the prior existence of a 'mother substance' postulated by Abel (1930). This phase of the problem cannot, however, be further pursued here.

SUMMARY

The experimental data on beef pituitary glands incubated at 37° C. for twenty-four hours do not support the conception that the relatively low oxytocic content in the pituitary glands of the great whales is caused by autolysis. Many factors influence the loss of activity of the hormones; namely, enzymes, hydrogen ion concentration, adsorption, incubation time and temperature. A method for the separation of the pressor and the oxytocic hormones has been demonstrated by the use of mixtures of acetone with methyl or ethyl alcohol. The fact that the hormones in powders made from autolyzed glands are much more soluble in absolute alcohol and acetone than the hormones in powders made from unautolyzed glands suggest the possibility that these hormones may be derived from a larger molecule.

The author wishes to extend thanks to Professor E. M. K. Geiling for his guidance and many suggestions throughout this investigation, and to Dr. E. F. Pike of the Armour Laboratories for a very generous supply of material used in this work.

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NOTES AND COMMENTS

DIETARY FATTY LIVERS IN MICE AND SENSITIVITY TO EXOGENOUS ESTROGEN

It is now fairly well established that detoxification of estrogen takes place in the liver (Zondek, 1934; Israel, *et al.*, 1937; Golden and Sevringhaus, 1938; Selye, 1940). Considerable evidence has accumulated recently concerning the increased sensitivity of rodents to endogenous or exogenous estrogens with the development of liver damage (Talbot, 1939; Pincus and Martin, 1940). Much of this evidence is derived from observations on animals to which substances have been administered which have a high degree of toxicity for liver tissue. The accumulation of fat in these livers is conceded to accompany a generalized pathologic degeneration of functional liver tissue. Whether the accumulation of fat *per se*, or whether the destruction of hepatic cells, or both, are the factors involved in increased estrogen sensitivity is not known.

It was considered advisable, therefore, to study sensitivity to exogenous estrogen in animals in which fatty livers, uncomplicated by irreversible pathologic processes had been produced by diet.

Approximately 90 mice of the Missouri strain, weighing over 20 gm. each, were ovariectomized. They were allowed to recover for 10 days, and to eat a Purina fox chow diet, henceforth called the stock diet. The absence of cornified cells in the vaginal smears in all the animals attested to the completeness of the ovariectomy. The mice were then divided into 2 equal groups. *Group A* was given a high fat-low protein diet,* a procedure which produces fatty livers in mice (Best, *et al.*, 1932). *Group B* was continued on the stock diet. Body weights were recorded at weekly intervals. Eight days after the institution of the fat diet in *group A*, 3 animals from each group were autopsied for analysis of total liver fatty acids and non-saponifiable material. Liver fat was determined by saponification, acidification and extraction with petroleum ether. The livers of the animals from *group A* appeared fatty on macroscopic examination, and had a total fat content which averaged 29 per cent. The livers of the animals on the stock diet (*group B*) were normal in appearance and contained an average of 6.5 per cent total fat. It was concluded that the fat diet was effective in producing fatty livers in *group A*.†

After initial priming of all animals, estrogen was administered as estrone in 10 per cent alcohol solution in 3 divided doses once per week for 4 weeks. Full vaginal cornification observed 48 hours after the injections was considered a positive reaction. The results are shown in table 1.

It would appear from these results that administered estrogen was of similar effectiveness in both groups until the fourth week of the experiment, at which time a decrease in sensitivity appeared to occur in *group A* receiving the high fat diet.

When sensitivity of the two groups is considered in relation to weight loss, there appears to be a correlation which may be responsible for the divergence in reactivity at the fourth week. The group on the fat diet lost an average of 4.1 gm. (0 to −10 gm.). The animals on the stock diet lost an average of 1.5 gm. (+1 to −4 gm.).

This reduced reactivity suggests that reduction of tissue capacity for estrogen stimulation may be a reflection of a lowered nutritional status of the animal. The animals on the high fat

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* Composition of the diet: sucrose, 45%; crisco, 40%; casein, 5%; yeast, 4%; salt mixture, 4% (Hubbel, R. B., L. B. Mendel, and A. J. Wakeman: *J. Nutrition*, 14, 273, 1937); cod liver oil, 2%.

† Samples for liver fat taken later in the experiment were inadvertently lost. The gross appearance of the livers in the fat-diet animals was fatty at these times, however.

TABLE 1. EFFECT OF FATTY-LIVER-PRODUCING DIETS ON THE SENSITIVITY OF MICE TO INJECTED ESTRONE, AS INDICATED BY VAGINAL SMEARS

VAGINAL SMEARS	END OF 2ND DAY	10TH DAY		17TH DAY	24TH DAY
	TOTAL ESTRONE, 0.15 μg.	TOTAL ESTRONE, 0.068 μg.[1]	TOTAL ESTRONE, 0.15 μg.[2]	TOTAL ESTRONE, 0.15 μg.	TOTAL ESTRONE 0.15 μg.
		Fat Diet			
Positive	31	4	4	20	14
Negative	14	24	9	18	24
Total No.	45	28	13	38	38
% Positive	69	14	31	53	37
		Stock Diet			
Positive	29	4	5	23	27
Negative	14	23	8	17	13
Total No.	43	27	13	40	40
% Positive	67	15	38	57	68

[1] This group consisted of animals which had shown a positive reaction to the dose a week before.
[2] This group consisted of animals which had shown a negative reaction to the dose a week before.
In all other cases, all the animals as a whole were given the same test dose without reference to previous reaction.

diet, therefore, became less sensitive to estrone than the controls, as the former tended to lose more weight. This is in line with previous observations (Evans and Bishop, 1922) regarding the effect of inanition on sensitivity to endogenous estrogen.

However, the experiment was not designed to investigate the effects of inanition on estrogen sensitivity. The conclusions regarding the effect of fatty liver on estrone sensitivity are therefore drawn from the data for the first three periods studied, during which time the weight losses of the two groups did not differ greatly. The striking similarity in reaction during this time permits the conclusion that the liver mechanism for detoxification of estrone is not impaired by dietary fat accumulation in that organ.

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EXCRETION OF ESTROGEN IN THE BILE

IN A RECENT publication, Heller and Heller (1943) refer to the report from our laboratory (Cantarow, *et al.*, 1942a) of the excretion of estrogen in the bile in a manner which indicates that the true significance of our findings was overlooked. They state that these authors (Cantarow, *et al.*, 1942a)

'found no estrogen in the bile of untreated dogs, but recovered 40 to 48 per cent of a huge intravenous dose of estrone (250,000 I.U.) from the common duct.' Their conclusion that estrogens are not rapidly destroyed or inactivated by the liver is open to question because of *a*) the unphysiological doses, *b*) the

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fact that the recovered estrogen may have been the more potent α-estradiol and *c*) by their failure to find estrogens in the bile of normal dogs.

This isolated quotation from the abstract of our presentation at the 1942 meeting of the Association for the Study of Internal Secretions is misleading. The following statements occur in the same abstract and in a report published in April, 1942 (Cantarow, *et al.*, 1942b):

If no bile was re-fed, approximately the same quantity (40–48%) was excreted (in the bile) in the second 24 hours. Following a single intravenous dose of 250,000 I.U. of estrone in alcohol, the level of blood estrogenic activity after 4 hours is about 10% of that at 10 minutes, falling to practically zero after 24 hours.

The significant fact is that enormous amounts of estrogen are excreted in the bile *after* the first 24 hours, *i.e.*, at a time when very little is present in the systemic circulation and in the urine. Moreover, in the paper read at the meeting and published in November, 1942 (Cantarow, *et al.*, 1942c), it was reported that recoveries of equal magnitude (90–95% in 48–72 hours) were obtained with 250,000 I.U. of α-estradiol and 10,000 I.U. of estrone intravenously and also with 250,000 I.U. of estrone subcutaneously, significant amounts being excreted during the third day. These observations with α-estradiol have since been confirmed repeatedly. In the discussion that followed presentation of these data, it was stated also that we had found large amounts of estrogen in the bile of women in late pregnancy and the puerperium. It appears to us that these observations justify the conclusion that estrogen may be excreted in the bile under physiologic conditions and, as stated in the abstract (Cantarow, *et al.*, 1942a and referred to by Heller and Heller, 1942), 'throw some doubt upon the hypothesis of *rapid* destruction or inactivation of estrogens by the liver in intact animals.' The findings following administration of α-estradiol eliminate the possibility, raised by these authors, of excretion in a form more active than that administered, at least insofar as present knowledge of estrogenic substances is concerned.

It may be pertinent to add that data now in press (Proc. Soc. Exper. Biol. and Med.) reveal the following: *a*) the action of estrone or of α-estradiol is not potentiated by addition to bile. *b*) The estrogenic activity of liver bile ('C' fraction), obtained by duodenal intubation of women at full-term pregnancy was found to be about 3 times that of the blood, and estrogen was demonstrated in relatively large amounts in the bile of women up to the seventh day *post partum*, the blood estrogen values during this period being relatively very low. *c*) Injection of chorionic or pregnant mare serum gonadotropins into bile-fistula dogs was followed by increasing excretion of estrogen in the bile (up to 700 I.U. daily) for the subsequent 4 days. *d*) After splenic implantation of a 15-mg. pellet of α-estradiol into a bile-fistula dog, estrogen was excreted in the bile (up to 2025 I.U., daily) for 23 days, none being demonstrable in the urine during this period.

On the basis of these observations, we feel that, for the dog, at least, and perhaps also for the human female, the conclusions are justified that *a*) the bile is an important medium of excretion of both endogenous and exogenous estrogen; *b*) there is serious doubt of the validity of the hypothesis that estrogens are *rapidly* inactivated or destroyed by the liver in the intact animal. It is not and has not been denied that the liver is *capable* of inactivating estrogens, but that it does so *rapidly* in the intact animal is open to question.

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NEW BOOKS

SEX HORMONES. BIOLOGICAL SYMPOSIA, VOL. IX. Edited by F. C. Koch, Frank P. Hixon Distinguished Professor and Chairman of the Department of Biochemistry of the University of Chicago, and Philip E. Smith, Professor of Anatomy, College of Physicians and Surgeons, Columbia University. Lancaster, Pa., The Jaques Cattell Press. $2.50. x and 146 pp. 1942.

The present volume is a continuation of the series of biologic discussions which have been concerned with such notable and widely varied subjects as The Cell Theory, Problems of Speciation, Muscle, and Visual Mechanisms. The present symposium takes up two principal aspects of the sex hormones.

In the first section, entitled Sex Hormones—Their Actions and Metabolism, a short introductory chapter by Carl Moore on the General Biology of the Testicular and Ovarian Hormones is followed by a discussion of metabolic influences of the sex hormones by A. T. Kenyon. This portion of the book is completed by chapters on the metabolism of estrogens by E. A. Doisy and the metabolism of male sex hormones by F. C. Koch.

The second section, entitled Hormonal Factors in the Inversion of Sex, is introduced by a discussion of inversion of the plumage of birds, by C. H. Danforth. The last three chapters all deal with experimental modifications of sex and sexual characters by steroid hormones. Experimental sex reversal in amphibia is discussed by R. R. Humphrey, in the rat by R. R. Greene, and in the opossum by R. K. Burns, Jr.

The two aspects of reproductive endocrinology selected for the scope of this volume are so widely disparate that they can be considered together only in the most general fashion. They are alike in that a great deal of work and thought have gone into both. The chapters by Kenyon, Doisy and Koch are concerned with careful evaluation of the results of chemical metabolic experiments. In them are considered the problems of formation, utilization and excretion of the sex hormones. To attack these problems, the classical approach by study of excretion products and of organ extracts has been adopted. Greene and Burns have followed a similar course in considering the problem of the induction of sex reversal by hormonal factors. Carefully planned experiments have shown what degree of morphological change in sex organs may be induced by suitably timed treatments with different amounts of hormones.

From the standpoint of provocativeness, the chemists appear to have outdistanced the biologists in this particular contest. Yet, the chemical data are suggestive rather than conclusive in many crucial points. For example, we have only the most incomplete story on the derivation of degradation products from the various precursors present in these regions of the body where inactivation or utilization occurs. On the other hand, the morphologic data are more self-limiting since they do not suggest immediately experiments which would reveal the mechanisms of the observed reactions.

The juxtaposition of two such widely differing sets of data leads to the reflection that extreme dichotomies exist in the field of endocrinology. In the last two decades endocrinology has matured into a specialty in the biologic field. Are we not now creating specialties within a specialty to such a degree that little if any contact remains between, say, the anatomical and the chemical aspects of the subject? If so, one may question the advisability of continuing unchecked this tendency. In the present volume it seems clear that each of the groups could have gained immeasurably by the knowledge and know-how of the other. It is surely advisable to study the changes in composition of substances within the cell itself, since this site is almost certainly the place where transformations occur. Yet, for want of the anatomist's lore on the manipulation of cells, the chemist is forced to resort to more indirect methods. Similarly, an understanding of the chemical events occurring within the cell would be of

obvious advantage to the anatomist in his search for the effect of hormones upon the visible structures.

It may be objected that such histochemical treatment has not been attempted because of the inherent complexity of the subject. To this the rebuttal may be made that many observations already exist which point the way. The destruction of steroid hormones by a thermolabile liver extract, and the prevention of such effects by cyanide poisoning, indicate directly the importance of such studies. The histochemical demonstration of cell inclusions in the adrenal and testes, the solubilites and reactions of which are similar to the same properties of ketonic steroids, points to at least one line of attack. Lastly, the collection of sufficiently large quantities of cellular parts for standard chemical analyses proves the alternate possibility of applying the micro-method to the cell rather than to the chemical reaction.

Despite these criticisms of the attitudes which are at present dominating research in this field, the symposium is a valuable one. Too little opportunity is offered and too little encouragement is given attempts to systematize large bodies of evidence. Even if one takes the view that suggestions for future research, which accrue in the course of such systematization, are frequently too circumscribed and specific, surely no better opportunity can be provided for the cultivation of more fruitful approaches than by the association of diverse minds assembled for the discussion of common topics of interest. In that it provides such opportunities the present symposium is a success.

EDWARD W. DEMPSEY

DIE THROMBOZYTEN DES MENSCHLICHEN BLUTES AND IHRE BEZIEHUNG ZUM GERINNUNGS- UND THROMBOSEVORGANG. A. Fonio, *Prof. Dr. med., und Prof. für Chirurgie an der Universität Bern,* UND J. Schwendener, *Dr. Med., Assistenzarzt.* Cloth. gb. Fr. 9.60. 130 pages (octavo); 112 figures. Bern, Hans Huber, 1942.

This volume is almost in atlas form, abundantly illustrated, containing 55 pages of figures; but the text is not limited entirely to a running commentary on the illustrations. Practically all of the observations on the thrombocytes[1] (platelets) were made on human blood by dark field microscopy and the illustrations consist chiefly of photomicrographs, excellently reproduced. In all studies *in vitro* the red blood corpuscles and leucocytes were separated from the blood by centrifuging, leaving only the platelets in the plasma.

The first section deals with the morphologic changes and behavior of thrombocytes held in plasma in which coagulation had been permanently inhibited by chemical means (usually 14 per cent solution of $MgSO_4$, 2 parts; blood, 7 parts). Most of the second section is devoted to the behavior of thrombocytes during clotting. The sequence of morphologic events was followed when clotting was initiated, after being temporarily inhibited by physical means (depressing the temperature to 1.0°C.). Brief reference is also made to the induction *in vivo* of small white thrombi in the capillaries of frog's tongue following diathermic injury of the endothelium by means of microelectrodes. This entire study is limited to microscopic observations. The authors do not attempt any critical analysis of the theories of blood coagulation.

When coagulation was permanently inhibited the changes in the physical state of the thrombocytes could be followed without the complication of fibrin formation. Active surface changes including extrusion of cytoplasmic processes was observed especially after contact with the glass of the slide or cover slip or with other thrombocytes. It is impossible to abstract the detailed descriptions of pseudopodial activity under varying conditions and periods of time, utilized in this study. Active, resting and transitional types of thrombocytes with characteristic behavior patterns were recognized. Formation of cytoplasmic vesicles which are gradually separated from the cytoplasmic surface to lie free in the plasma was observed in older preparations.

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[1] The term thrombocyte is uniformly used to designate the so-called third element of mammalian blood, the thromboplastid or platelet. When reference is made to the comparable nucleated element of the blood of other vertebrates the term 'spindle cell' is used.

The authors conclude that the platelet is comparable to a cell, with the granules (granulomere) functioning as a nucleus, and that the thrombocytes are active participants in the coagulative process. The morphologic components of the thrombocytes and the physical changes occurring in them are definitely correlated with recognized stages in the formation of the clot. The cytoplasm (hyalomere), with induced pseudopodial formation, functions in adhesion and agglutination under the thromboplastic influence of contact *in vitro* with uneven surfaces of the glass slide or cover slip. Similar responses are invoked *in vivo* on contact with injured endothelium of a blood vessel or on the margins of a wound.

The granules are the bearers of thrombokinase and function on the breakdown of the cytoplasm. They are primarily responsible for the initiation of fibrin formation and in the production of the fibrin network. They are also secondarily involved in the subsequent retraction of the clot since eventually they constitute the entire coagulation centers from which the fibrin threads radiate. With the massive extrusion into the plasma of cytoplasmic vesicles from the bodies of the platelets ('frog-spawn effect'), the volume of the individual coagulation centers (single thrombocytes or clusters of thrombocytes) is rapidly reduced and the consequent shrinkage of these numerous microscopic centers results in the macroscopic retraction of the clot.

A limited bibliography and an adequate index complete the volume.

A. B. Dawson

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Morton, R. A.: The Application of Absorption Spectra to the Study of Vitamins, Hormones and Coenzymes. 2nd ed. 10 tables, 80 text figures and 7 plates. Pp. 208. Adam Hilger, Ltd.

Nedzel, A. J.: Vascular Spasm, Experimental Studies. 161 figures. Pp. 151. University of Illinois Press. $2.75 clothbound, $2.25 paperbound.